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THE DEVELOPMENT OF THE ENZYME LINKED
IMMUNOSORBENT ASSAY (ELISA) FOR SCHISTOSOMIASIS
AND ITS EPIDEMIOLOGICAL APPLICATIONS

A Thesis submitted for the degree of
Doctor of Philosophy in the
University of London (Faculty of Medicine)

by

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The Development of the Enzyme Linked Immunosorbent Assay (ELISA) for Schistosomiasis and its Epidemiological Applications

by Moira L. McLaren

ABSTRACT

The use of immunodiagnostic techniques for studying epidemiological aspects of schistosomiasis have been of limited value in the past owing to the type of techniques adopted and the wide cross reactivity exhibited by antigens used in the tests. In this study a relatively new technique, the Enzyme Linked Immunosorbent Assay (ELISA) was used. After the development of a standardised procedure it was applied to a wide range of S. mansoni infections representative of different epidemiological situations.

A number of modifications in the basic enzyme assay technique were introduced and their merits discussed. Those which proved to be of value were a) the use of ultracentrifuged antigens, b) the development of a method for the determination of the optimum value for the reference serum endpoint, c) the use of serum eluted from filter paper blood spots, d) the use of precoated antigen plates prepared by air or vacuum drying and e) the development of quality control procedures.

In evaluating the diagnostic performance in human schistosomiasis ELISA was compared with conventional immunodiagnostic techniques such as skin tests, complement fixation tests (CFT), indirect fluorescent antibody tests (IFAT) and a recently developed radioimmunoassay (RIA) incorporating a species specific egg antigen. Sera from E. Africa and the West Indies were used to assess the test in relation to important diagnostic criteria. The ELISA using soluble egg antigen exhibited higher levels of sensitivity and specificity compared to skin tests, CFT and IFAT with sera from schistosome endemic and controls from non-endemic areas. Unlike the RIA however cross reactions occurred with heterologous schistosome infections and the test did not show the same degree of association with intensity of infection and responsiveness to chemotherapy.

In two large control programmes, in the Gezira region of the Sudan where S. mansoni is hyperendemic, and St. Lucia where prevalence is low, an evaluation of the diagnostic reliability of the test and the relevance of antibody measurements was made in two widely different epidemiological situations. The relative merits of a serological as compared with a conventional parasitological approach in epidemiology were examined with particular emphasis on the use of serodiagnosis for measuring incidence rates and as a primary screen for the selective removal of uninfected individuals in a prevalence survey and prior to chemotherapy.

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CHAPTER I

INTRODUCTION

1. Epidemiological aspects of schistosomiasis

Schistosomiasis is probably one of the earliest parasitic infections to be recorded, evidence for its existence dating back to the early Egyptian civilizations of the 20th century BC as revealed in their hieroglyphics, (Ansari, 1973). It was not, however, until the late 19th and early 20th centuries through the discoveries of Bilharz in 1852, Katsurada in 1904 and Sambon in 1907 that the three major species of human schistosomiasis, S. haematobium, S. japonicum and S. mansoni respectively were identified and the complexities of their life cycles unravelled. Today schistosomiasis is a common infection in the tropical world, primarily affecting the rural poor it is estimated that some 600 million people are exposed to infection with over 200 million infected, Andreano et al. (1974). The parasite exhibits a wide global distribution, S. mansoni is distributed throughout large parts of Africa, parts of Central and South America and the West Indies; S. haematobium has a more restricted distribution being confined to the African continent and S. japonicum is the only species to occur in the Far East, mainly China and the Philippines. A fourth species S. intercalatum is of less importance being restricted to several small foci in Africa.

Schistosomiasis is becoming an increasing public health problem in many developing countries particularly where irrigation schemes are being implemented or expanded as in the case of Volta Lake in Ghana, the Kariba dam scheme in Zambia and the irrigation canal systems in Egypt and the Sudan. While these schemes have undoubtedly brought enormous benefits in improved agricultural opportunities, they have in turn provided abundant new habitats for the intermediate snail host of the parasite and this has led to an increase in infection in the human population.

The patterns of infection within exposed human communities have been established from a number of detailed cross sectional and longitudinal surveys.

A cross sectional survey in a region of Brazil (Kloetzel,

1963) showed infections to be acquired at an early age with prevalence (numbers infected) reaching a peak of 80% by the age of 10 years. In older age groups prevalence remained at a high level although the intensity of infection (parasite load) declined between the ages of 15-30. A similar pattern of infection was observed in a cross sectional study in Uganda (Ongom & Bradley, 1972). Although prevalence was nearly 100% in all age groups there was some reduction in intensity from peak levels in the 10-14 age group.

The relationship between prevalence (endemicity) and intensity of infection was studied in a cross sectional survey of infected people in St. Lucia (Jordan, 1972). The study revealed that where the prevalence rate was under 50% there was no difference in egg output levels (intensity) between groups aged under 20 and those aged over 20, but in areas of higher prevalence, particularly over 75%, there was a notable difference in intensity with the under 20 year group where egg excretion rates were almost twice those of the adult population.

Recently, Omer *et al.* (1976) carried out a cross sectional prevalence and intensity survey of a stable farming community living in the Gezira region of the Sudan where S. mansoni infection is hyper-endemic. Prevalence reached a peak of over 80% by the age of 20 years and thereafter declined to about 50%. The intensity profile showed a similar age specific decline.

Community studies in areas endemic for S. haematobium have revealed very similar patterns of infection. Bradley and McCullough (1973) carried out a detailed cross sectional survey in an area of Tanzania highly endemic for S. haematobium. In the cross sectional survey peak prevalence occurred in the 5-9 age group and was over 90%, thereafter it declined to under 40% in the over 50 age group. The arithmetic mean egg output also showed a very marked decline from the age of 10 years.

The results of these and other surveys have shown that within the host population the distribution of adult flukes appears to be overdispersed rather than randomly distributed (Bradley & May, 1978). Crofton (1972) argues that one of the features of parasite populations despite their phylogenetic diversity is their tendency to show an overdispersed distribution in the host population and that this is a

fundamental factor in the maintenance of a dynamic equilibrium between host and parasite. As a consequence of overdispersion only a small percentage of the infected human population harbour very large numbers of adult worms. In many endemic areas this may be less than 10% and even in hyperendemic regions only 30% of the population may have very heavy infections (Cheever *et al.*, 1968, 1977). This is also reflected in the levels of egg output in the community. McCullough & Bradley (1973), found that 10% of infected children were excreting 60% of the eggs, and studies in Brazil (Lehman *et al.*, 1976) revealed a similar pattern, where 6% of the population infected with S. mansoni excreted over 50% of the ova. This type of parasite aggregation has important consequences with respect to the development of disease, since in both S. mansoni and S. haematobium infection there is a well established relationship between the intensity of infection expressed as egg output and the presence of overt clinical disease (Sion-Arap *et al.*, 1976; von Lichtenberg *et al.*, 1971).

Another important feature of parasite distribution in the host population is the relationship between prevalence of infection and age. Prevalence of schistosomiasis appears to be age related particularly in S. haematobium with peaks of infection occurring in children and adolescents and declining in the adult population. Similar age specific declines are found in relation to intensity of infection. In areas of low endemicity (reduced transmission) there is a shift in the peak prevalence and intensity to older age groups and there is generally a far less marked decline in both prevalence and intensity with age.

The characteristic age specific decline in prevalence and intensity of infection, particularly in hyperendemic regions has been attributed to two phenomena, firstly the development of a specific acquired resistance to reinfection and secondly, reduced water contact with age resulting in less exposure to reinfection.

Evidence in support of the first hypothesis has been based largely on the observed decreases in intensity of infection with age as measured by egg output and an apparent stability of egg excretion levels in children over several years suggesting that superinfection does not occur. In a longitudinal survey of S. haematobium infected children, McCullough & Bradley (1973) studied the stability of egg output. Although large variations in egg count were observed over a period of

several weeks egg counts tended to show relative stability over a period of three years, in that a group which were heavy egg excretors in the first year of the study were still in that category three years later and similarly for the low intensity excretors. On the basis of these data and those obtained from a cross sectional study (Bradley & McCullough, 1973) three phases of infection pattern in schistosomiasis were postulated. Phase one involved the acquisition and increase of infection; phase two a decrease in worm load combined with concomitant immunity and phase three, a steady state with the loss of immunity and reinfection balancing in later life.

Evidence for the development of an acquired resistance in S. mansoni infection is somewhat less substantial. Jordan (1974) carried out a longitudinal survey of infected children in St. Lucia to look at the stability of egg excretion patterns. He found that egg counts tended to increase in the first five years of infection, but subsequently became more stable, this was most pronounced in children with low intensity infections. In heavily infected children the changes were far less apparent over this period of time.

It is clear from these data that there is some epidemiological evidence for the development of an acquired resistance to schistosomiasis in man, but it is not conclusive and where it does develop the process would appear to take several years and may be dependent on levels of re-exposure and intensity of initial infection.

There is also evidence to suggest an alternative though not exclusive hypothesis, that the decline in prevalence and intensity with age is due to reduced water contact. Warren (1973), argues that frequency of water contact is strongly age related, being greatest in children, and that this could account in the main for the observed age specific decreases in both prevalence rates and intensity of infection.

2. The role of diagnostic methods in epidemiology

In community studies diagnostic methods are used a) to establish the existence of an endemic focus by detecting infected individuals, b) to determine the numbers infected and severity of infection for the purpose of assessing the problem in an area, c) to

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collect data useful for the evaluation of a control programme and d) to collect evidence from infected individuals about the course of infection and the effects of therapy. This information is based on the measurement of three epidemiological parameters in the human population.

Prevalence is a measure of the proportion of people in the population infected at a given time and related to the numbers of persons at risk during that period. It is usually expressed as a rate and in schistosomiasis is age related. Incidence, is a measure of the rate at which uninfected (susceptible) individuals in the population become infected and is related to the number of persons at risk during that period (e.g. 1 year). Intensity is a measure of the parasite load in the population, and is again often age related (Ansari, 1973).

Prevalence, incidence and intensity are particularly important for measuring the progress of a control scheme by assessing the effects of its impact on man through the measurement of human infection as opposed to disease, in other words looking for changes in the rate of transmission of infection. Changes in the rate of transmission are most rapidly detected by measuring the incidence of infection, the rate at which uninfected people become positive. The incidence can be calculated from the occurrence of infection in a cohort of residents known to be uninfected previously. In endemic areas children provide the most useful group because incidence is highest and there are not the problems of acquired resistance which might occur in older age groups. Incidence is the only reliable indicator when chemotherapy has been used as a method of control since the reduced prevalence following effective treatment does not necessarily reflect reduced transmission. If the control programme is successful then as the incidence rate falls the prevalence also begins to fall as the age groups fail to be infected and infections already acquired begin to die out. Changes in infection intensity in the younger age groups will also provide a sensitive reflection of changes in the intensity of transmission, but to be reliable standardised techniques have to be used for all surveys before and after control measures have been implemented.

It is clear from the preceding section that measurements of important epidemiological parameters in human schistosomiasis have been

based largely on the results of parasitological diagnostic methods which depend on the recovery and identification of ova from stools or urine of people infected with S. mansoni and S. haematobium respectively.

The parasitological methods most commonly used in epidemiological surveys include concentration techniques such as the Merthiolate Iodine Formol Concentration (MIFC) (Richie et al., 1948), the direct Kato smear technique (Kato & Miura, 1954) and the filtration staining technique (Bell, 1963) for S. mansoni infection, and sedimentation and filtration techniques for S. haematobium (Peters et al., 1977). These techniques have been used in both qualitative and quantitative studies, but few really detailed comparative assessments have been carried out to establish relative sensitivity and reproducibility and their quantitative reliability.

Of the techniques used for the diagnosis of S. mansoni infection the Kato technique has probably had widest investigation. Martin & Beaver (1968), carried out a study on the sensitivity and reproducibility of egg counts with the Kato thick smear technique, which involves the principle of direct faecal sampling. They found the technique to have a comparable level of sensitivity with reports on the Bell filtration technique, and also found egg counts to be fairly uniform, although the data were not critically analysed.

Teesdale & Amin (1976) carried out a comparative assessment of the Bell technique, the modified Kato technique and a digestion technique for field diagnosis of S. mansoni. The modified Kato and Bell technique had comparable levels of sensitivity in detecting lightly infected patients, the most important group for determining this parameter, and had a higher level compared to the digestion technique.

In a recent study to examine the stability of egg counts in infected patients in Brazil, Baretto et al. (1978), observed large variations in counts between consecutive stool samples and found that using the Kato method only two thirds of patients finally categorised as heavy egg excretors were identified on their first examination.

Parasitological techniques have clearly been of great value in determining epidemiological parameters of human schistosomiasis,

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Parasitological techniques have clearly been of great value in determining epidemiological parameters of human schistosomiasis,

particularly in S. haematobium where the techniques for both qualitative and quantitative diagnosis are sensitive and reproducible. Detection of S. mansoni ova in stools poses greater problems, since large numbers may be retained in the tissues of the bowel and liver in the chronic stage of infection. Infections may also be missed when intensities are low as in early childhood and fall below the level of consistent detectability of the stool techniques. Kloetzel (1963), estimated that only 20% of the eggs laid may actually be eliminated in the faeces, and hence to detect light infections techniques have to be very sensitive.

In the light of some of the drawbacks inherent in parasitological diagnostic methods, particularly for S. mansoni infections, attention focused on the use of indirect methods for diagnosis. In the last sixty or so years a great deal of work has centred on the development of immunological approaches. These have been based on the measurement of hypersensitivity reactions to schistosome antigens, the detection of humoral antibodies and more recently schistosome antigens present in the urine or serum. The range of techniques which have been used is extensive and includes: intradermal tests for both immediate and delayed hypersensitivity reactions; serological tests based on an agglutination reaction such as, the indirect haemagglutination test (IHA), the bentonite flocculation test (BF) and the cercarial agglutination test (CA); tests based on a precipitation reaction such as, the circum oval precipitin test (COPT), around cercariae in the cercarienhiillenreaktion (CHR) and in gel medium immunoelectrophoresis (IEP/CIE); on the ability to fix complement (CF test); and in indirect labelled antibody assays such as the indirect fluorescent antibody test (IFAT), the defined antigen substrate spheres (DASS), radioimmunoassay (RIA), and more recently enzyme immunoassays (EIA/ELISA).

As the above list illustrates almost all the major immunological techniques developed have been applied to schistosomiasis. While several of these techniques have proved valuable analytical tools in basic studies on the immunology of schistosome infections, their record in the epidemiological field is perhaps less impressive.

It is felt by many parasitologists that developments in the immunodiagnosis of schistosomiasis have moved sideways rather than

Figure I. Criteria of a diagnostic test for schistosomiasis

ESSENTIAL NEEDS:

1. HIGH LEVEL OF SENSITIVITY
2. HIGH LEVEL OF SPECIFICITY
3. EASE OF SAMPLE COLLECTION
 - a). Use of untrained personnel
 - b). Socially acceptable method
 - c). Cost effective
4. EASE OF TEST OPERATION
 - a). Use of untrained personnel
 - b). Good test reproducibility
 - c). Cost effective

SECONDARY NEEDS:

1. QUANTITATIVE
 - a). Able to differentiate active from past infection
 - b). Responsive to intensity of infection
 - c). Responsive to chemotherapeutic treatment
2. SUITABLE FOR USE IN SMALL FIELD LABORATORIES

forwards, exemplified by a diversification in the range of techniques employed, but minimal progress in improving the quality of diagnosis. This somewhat pessimistic view is conveyed by Davis (1974) "to many physicians, chemotherapists or parasitologists, the concept of diagnosis of schistosomiasis by immunological methods is regarded with suspicion if not cynicism."

Before looking in detail at some specific applications of immunodiagnostic procedures in an attempt to assess whether such criticism is entirely justified, it is perhaps worth examining the basic criteria on which the quality of a diagnostic procedure is judged. Figure 1 illustrates some of the most important criteria of an immunodiagnostic test for schistosomiasis.

Levels of sensitivity and specificity in a test are of fundamental importance as they govern the quality of a diagnostic procedure. The type of information required in an epidemiological study will also influence the relative importance of these parameters; for example screening procedures should have a high level of sensitivity even at the expense of some specificity, whereas a survey which requires more definitive diagnostic information will require a test of high specificity. The area in which the survey is to be carried out can also be of importance. If a survey is carried out in an area where endemicity is low then the test requires a high degree of specificity since antibody prevalence rates may include a high proportion of false positive reactions. Small decreases in specificity greatly influence false positive rates. Conversely in hyper-endemic regions where antibody prevalence rates are high a slight loss in test specificity will not greatly affect the estimate of the true antibody prevalence rate.

Both the nature of the test system, that is the immunological principles on which its development is based, and the nature of the antigen preparations used in the test will determine the sensitivity and specificity, and to a greater extent they influence the other criteria. For example, the type of sample that can be used in the test, the degree of ease with which the test can be performed, and the reliability of the test system. This involves both reproducibility, acceptable matching on repeat tests of the same sample, and agreement,

the ability of different workers to obtain similar results for a given test on the same serum sample. The quantitative relationship of a measured immunological parameter to the intensity of infection, or the assessment of effective drug treatment are also influenced by the test procedure and the type of antigen used.

Intradermal tests which depend on the measurement of either immediate or delayed hypersensitivity reactions to soluble antigens have proved one of the most popular immunodiagnostic procedures for schistosomiasis. First used by Fairley & Williams in 1927, (Kagan & Pellegrino, 1961) the technique has since been applied to a wide range of epidemiological surveys and many of these have been covered in the detailed review of immunological methods by Kagan & Pellegrino (1961).

It is evident from many of the early studies that considerable variation in the diagnostic performance of intradermal tests exists, a point raised by Kagan & Pellegrino (1961), "the sensitivity of intradermal tests cannot be properly evaluated because of the diversity of the antigens and the choice of criteria for interpreting results together with the lack of uniformity among individuals in the groups to which the test has been applied".

The selection of appropriate antigens is a point raised by many workers in this field. Davies & Eliakim (1954), showed the importance of using homologous parasite extracts rather than extracts from the intermediate snail host. Using infected snail antigen in the test, sensitivity was 89% and specificity 73%, but this increased to 90% and 98% when adult worm antigen was used. Partial purification of antigens by acid precipitation or alcohol delipidisation has also been advocated to improve the quality of intradermal tests (Pellegrino *et al.*, 1957; Sadun *et al.*, 1958). The use of hypersensitivity responses as an indicator of effective treatment was also looked at in some of the early studies. Oliver-Gonzalez *et al.*, (1955a) observed minimal changes in positivity rates following treatment, even after several years, and concluded that the test was of limited application in this area.

Before looking in detail at some of the more recent applications of the intradermal test, it is perhaps worth citing some of the conclusions reached by Kagan and Pellegrino (1961), in their review of

the intradermal test as an immunodiagnostic procedure for schistosomiasis. They drew particular attention to the range of sensitivity and specificity reported in the test, related mainly to the antigens employed; also to the need for greater standardisation in the test procedure to improve the interpretation of results. They also mentioned its limitation for assessing chemotherapeutic treatment. Despite some of these limitations the intradermal test has continued to be used in epidemiological surveys, but almost always as an adjunct to parasitological diagnosis.

Moriearty & Lewert (1974) studied both immediate and delayed hypersensitivity reactions in Ugandan adults and children infected with S. mansoni. They examined levels of test sensitivity and specificity and the relationship of the hypersensitivity response to egg excretion levels. Using both crude worm and cercarial antigen extracts, sensitivity and specificity levels were in general poor. Positive responses were highest with immediate hypersensitivity reactions, 76-96%, but specificity was often as low as 45%. Although delayed responses were generally more specific, sensitivity (50%) was lower. One of the weaknesses of this study, recognised by the authors, was the use of parasitologically negative individuals living in the same endemic area to determine test specificity, since it is conceivable that some of these individuals may have been infected if repeated stool examinations had been done. No association in immediate hypersensitivity response was found between egg excretion levels or clinical manifestations. They concluded that the utility of skin tests for immediate or delayed hypersensitivity in diagnosis of schistosomiasis seemed limited. Exposure to cercariae of non-human schistosomes is a further limitation of the intradermal test. Individuals exposed to cercariae of non-human schistosomes develop marked hypersensitivity responses as the cercariae penetrate but fail to establish in the human host. These can lead to non specific reactions in an intradermal test and seriously distort the results of an epidemiological survey where human and animal schistosomes occur concomitantly. The significance of this has been discussed by Moore et al. (1968) and more recently by Lee et al. (1973).

A more recent evaluation of the intradermal test in an S. mansoni infected community in Puerto Rico again reveals the shortcomings of this technique (Hiatt et al. 1978). Using an S. mansoni adult worm antigen immediate hypersensitivity reactions were measured

in individuals of wide age range but with low intensity infections. It was found that the intradermal test did not accurately reflect infection status within the community, based on the results of stool examination. Only 36% of infected children reacted in the test and this increased to only 78% in adults. The problem of a persistent immunological response following loss of infection affected specificity in the adults. It was felt that the technique had limited epidemiological use and could only be combined with a parasitological survey.

What is very apparent from these more recent studies on the use of the intradermal test is the extent to which they reinforce the points made by Kagan & Pellegrino nearly twenty years ago. The use of the intradermal test in epidemiological studies still reveals differences in the levels of sensitivity and specificity, and while variation in endemicity of infection, intensity and prevalence of other helminth infections within different communities will influence these parameters, it is also a reflection of the lack of standardisation, particularly of antigen preparations that has prevailed in this area.

The present situation can, however, be viewed with some optimism as illustrated by the recent work of Senft & Maddison (1975) on the use of a specific proteolytic enzyme from S. mansoni for measuring hypersensitivity responses. Groups of infected children from St. Lucia and uninfected controls from St. Vincent were injected with a crude adult worm antigen extract and the purified enzyme antigen. Although sensitivity in the infected St. Lucian children was lower with the purified antigen, the specificity of responses was greatly improved. No cross reactions occurred in the St. Vincent control group and a lower proportion (6%) of the St. Lucian stool negatives reacted. The results of this study show a considerable improvement in the quality of skin tests compared to the results of an earlier comparative study by Warren et al. (1973), in similar groups of patients.

Several immunodiagnostic tests for schistosomiasis have been based on the principle of agglutination. The earliest of these was the flocculation test developed by Brandt & Finch in 1946 (Kagan & Pellegrino, 1961), following an earlier described test for trichinosis. The test is based on the binding of an antigen extract to insoluble

particles, cholesterol crystals as in the original study, or more recently bentonite particles or latex beads. The addition of specific antibody to the antigen coated particles then results in the formation of an immune complex causing the particles to clump or agglutinate together.

A detailed description of the technique is provided by Anderson (1960) who applied the cholesterol-lecithin slide test (CL), using delipidised cercarial antigen, for serodiagnosis of S. mansoni infection. Although the test proved sensitive, cross reactions were observed with patients infected with trichinosis. The problems of cross reactivity from unrelated helminth infections in this test is stressed by Kagan (1968) who recorded over 20% of false positive reactions in a study of children living in a non endemic schistosome area, but where ascaris infection was prevalent. Allain et al. (1972) used a modification of the technique in the form of bentonite particles as the inert carrier. This test (BF) was observed to have a lower level of sensitivity (57%) compared to the CL test (68%), but was more specific. Cross reactions were still observed mainly from patients with trichinosis or visceral larval migrans. Buck & Anderson (1972), in an epidemiological study to assess the validity of the slide flocculation test again observed poor specificity in this procedure. Although the test using cercarial antigen was sensitive for S. mansoni infection, cross reactions were particularly common in patients with ascaris and hydatid disease.

The property of viable cercariae to agglutinate in the presence of heat inactivated sera from infected animals lead to the development of another agglutination based test, the cercarial agglutination test, Liu & Bang (1950). This test has had virtually no epidemiological application, possibly because of poor results obtained with human sera, and the greater success of the CHR test which is based on a different immunological principle.

The most recent of the agglutination tests to be used in schistosomiasis was the indirect haemagglutination assay (IHA), which has proved a successful serodiagnostic procedure in a large number of virus and parasitic infections. Its first use in schistosomiasis was described by Kagan (1955) and Kagan & Oliver-Gonzalez (1958) using worm and cercarial antigen extracts absorbed on to tanned sheep

red blood cells. Although the sensitivity of the test was reported to be adequate problems were encountered with the reproducibility of end point titres. There have been mixed reports on the sensitivity and specificity of the IHA test when used on groups of human sera. Hoshimo et al. (1970) reported the test with adult worm antigen to have a sensitivity of 96% and a specificity of 97%, comparable to those obtained by Umaly et al. (1974) with levels of 90% and 100% respectively. In a comparative study of several serodiagnostic tests Allain et al. (1972) found the sensitivity of the IHA to be low, only 47%, below that of the BF (68%), CF (48%) and IF (57%). The low levels in their study may have been because the individuals screened had not resided for long in the endemic area and had therefore received repeated exposure to infection.

One feature of antigen antibody complexes is their capacity to form visible insoluble precipitates. This has been exploited in the development of immunological techniques several of which have been applied to the immunodiagnosis of schistosomiasis. Two tests which are based on this principle, the circum ova precipitin test (COPT) and the Cercarien-Hullen reaction (CHR) employ whole viable stages of the parasite life cycle as antigen. In the COPT live ova are incubated in patients' serum and streams of precipitate form from the reactions between specific antibodies and soluble egg antigen, which seeps through small pores in the egg membrane.

This technique which was initially developed by Oliver-Gonzalez (1954) has proved a most successful immunodiagnostic procedure. In the early studies it was observed that the secretory egg antigens were stage specific, since prior absorption of worm and cercarial antigen failed to prevent the formation of precipitate. Later work by Oliver-Gonzalez et al. (1955b) showed not only stage but species specificity as well. Using ova from heterologous species of schistosomes no precipitate was observed with infected patients' serum. These properties have made the COPT one of the most specific immunodiagnostic tests for schistosomiasis, and this undoubtedly accounts for its wide use in S. japonicum areas where concurrent infections with other trematodes such as clonorchiasis and paragonimiasis occur (Kagan, 1976). Work by Yagore et al. (1968) and Matsuda et al. (1977) also report the test to be highly sensitive when applied to epidemiological screening, with antibodies detected in up to 99% of infected

patients. In a more recent study in a low endemic area of Puerto Rico (Ruiz-Tiben, 1979) the test was reported to be highly sensitive detecting antibodies in 95% of infected patients, many with very low infection levels, as well as very specific.

In one of the early studies, Oliver-Gonzalez et al. (1955c) reported that the COPT had a useful role in the assessment of effective chemotherapeutic treatment. They observed a marked decrease in the intensity of the reaction some 17 weeks after treatment with a conversion to negative in cured patients after six months. Similar findings have been reported from other studies (Shoeb et al., 1967; Mello et al., 1979).

The CHR technique which was developed somewhat earlier in 1949 by Vogel & Minning (Kagan & Pellegrino, 1961), is based on the same principle and involves the formation of an antigen antibody precipitate around viable cercariae incubated in infected patients' sera. Initial studies on its application for diagnosis showed it to be sensitive and specific although it did not exhibit the degree of stage or species specificity of the COPT (Kagan & Pellegrino, 1961). Some more recent figures of sensitivity and specificity for this test are provided by Umary et al. (1974) in a comparative study of the CHR with the IHA, IFAT and CFT. This study revealed the CHR to have a sensitivity rate of 100% and 98% in proven cases of S. mansoni and S. haematobium infection respectively, with only 2% of false positive reactions in uninfected controls. These levels were higher than those obtained for the other tests.

Although both these techniques continue to be used they are technically demanding as skill is required in correctly interpreting the precipitin patterns and there are drawbacks with the need to preserve the viability of ova and cercariae for use as antigen.

The second group of immunological methods which are based on precipitation are the gel diffusion or immunoelectrophoresis (IEP) tests. Both employ soluble antigen extracts, with the formation of a visible precipitate in the gel after diffusion of antigen and antibody in the first test, or following electrophoretic migration of antigen and or antibody in the second. While both of these techniques have proved invaluable analytical tools, they have not been widely used as

immunodiagnostic procedures. The main drawbacks in both methods for schistosomiasis are the requirement for high concentrations of antigen, and a generally low level of sensitivity in detecting antibody in infected patients. Scapin & Tendler (1977) reported the sensitivity of gel diffusion to be about 54%, and Deelder *et al.* (1975) reported sensitivity in the IEP to be 88%. A modification of the conventional IEP system is the counter current immunoelectrophoresis test (CIE) where soluble antigen migrates in the gel under the effect of the electric field in one direction and antibody in the other direction by the effects of electroendosmosis. This system is somewhat more sensitive than gel diffusion or IEP and much more rapid (Draper, 1976). The chief application of this technique has been in the detection of circulating antigen in schistosomiasis, a subject which will be mentioned later.

"With the proper antigen the complement fixation test is probably the best serological procedure for diagnosis of schistosomiasis", a comment made by Kagan (1968), on the earliest of the immunological techniques used for diagnosis of schistosomiasis.

Its initial development at the turn of the century owed much to the efforts of Japanese workers Yoshimoto in 1910 and Hayani & Tamaha in 1910 (Kagan & Pellegrino, 1961). This was followed later by the work of Fairley in 1919 who applied the technique to the serodiagnosis of *S. mansoni* and *S. haematobium* infection. Fairley's work was of interest in that he strongly advocated the use of alcoholic or aqueous extracts from the intermediate snail host of the parasite as a source of antigen for use in the test, an approach which was subsequently adopted by many workers. The impact of this was in the view of Kagan & Pellegrino (1961), to lead to an inhibition in the developments of new antigens for use in the test. In fact it was not until thirty years later through the work of Chaffee *et al.* (1954), that a marked change in both the type of antigens and technique occurred. Their major contribution was firstly the development of an ether delipidisation procedure for isolating adult worm antigens, and secondly improvements in the quantitative basis of the technique. In their study test sensitivity was 98%, a considerable improvement on those tests employing snail antigen extracts where sensitivity was sometimes as low as 54% (Davies & Eliakim, 1954). The delipidisation process also removed substances which had previously caused non specific

fixation with sera from cases of syphilis, a major cause of false positive reactions in previous CF tests. The Chaffee antigen as it became widely known continued to be used in the CF test although there were several attempts to further improve the quality of the antigens used (Schneider et al., 1956; Sleeman, 1960). A detailed examination of different antigen preparations was carried out by Rifaat & Khalil (1965). Using appropriate groups of sera from schistosome infected patients and uninfected controls, sensitivity of the CF test ranged from as low as 10% with a lipoid F. gigantica antigen to 99% with a Chaffee extract of S. mansoni and a crude aqueous extract.

The application of the CF test in epidemiological surveys has also continued to receive detailed study. Buck & Anderson (1972) carried out an evaluation of its diagnostic performance in samples from two endemic areas of Chad and Ethiopia, and controls from non endemic areas of Peru and Afghanistan. The test with adult worm antigen gave high specificity, but low sensitivity in children, and chronic infections. High specificity in the test was reported by McCarten & Nzelibe (1975), when the CF was used as a supplementary screening procedure for overseas personnel returning from schistosome endemic areas. Although it was less sensitive than the indirect fluorescent antibody test, far fewer non specific reactions were reported with other helminth infections. Although still a popular procedure in some laboratories the CF test has since been superseded by the most recent group of immunological tests, the indirect labelled antibody techniques. The first of these to be developed for schistosomiasis was the indirect fluorescent antibody test IFAT.

The use of the IFAT for serodiagnosis of schistosomiasis was first described by Sadun et al. (1960) using cercariae as the source of antigen. Since its development the technique has been used in a wide range of epidemiological surveys. Using sera in the form of filter paper blood eluates, collected from different endemic areas (Sadun et al., 1961) antibodies were detected in 92% of confirmed S. mansoni cases. The test was also observed to have good specificity with cross reactions occurring in only 6% of patients with other diseases. This category did not however include patients with other helminth infections which might have led to a higher level of non-specific false positive reactions in the test.

A later study (Kagan et al., 1965) found the test to be less satisfactory. Antibodies were detected in only 75-80% of infected patients and a high proportion of false positive reactions (40%) occurred in cases of trichinosis and filariasis. These were in Eskimos and South Pacific islanders from non endemic schistosome areas. Their study also drew attention to the problems of cross reactivity in the test from persons exposed to avian or animal schistosomes. Sadun & Biocca (1962) observed strong cross reactivity in both the IF and intradermal tests in individuals who had been exposed to cercariae of S. bovis on the island of Sardinia. Improved levels of sensitivity were reported by Coudert et al. (1967) and later by Umaly et al. (1974) using cryostat sections of adult worms embedded in tissue. Using this type of antigen test sensitivity was of the order of 95%, but cross reactions still occurred with sera from uninfected individuals.

One of the interesting epidemiological applications of the IFA test was described by Schiff & Yianakkis (1976). They carried out a cross sectional prevalence survey for S. mansoni infection in groups of adults and children living in several communities in Rhodesia with widely differing transmission rates. In children a strong association was observed between antibody levels, and prevalence of infection. This difference was far less apparent in adults since antibody levels probably reflected the effects of repeated exposure. The study concluded that children provided a good indicator group for measuring the transmission of schistosomiasis and that antibody levels could be used to indicate a changing situation in response to a disease control programme.

Although most commonly used with tissue fixed sections of parasite, two fluorescent tests using soluble antigen extracts have been described, the soluble fluorescent antibody test (SAFA) by Sadun & Gore (1967) and the defined antigen substrate spheres (DASS) by Deelder et al. (1974).

The enzyme and isotopic assays were the most recent of the labelled antibody techniques to be applied in schistosomiasis. Although popular in many fields isotopic assays have not had widespread use as immunodiagnostic procedures in schistosomiasis. Williams et al. (1971) reported the use of a radioactive antigen microprecipitation technique

assay (RAMP) for detecting antibodies, by their capacity to bind I₁₂₅ labelled antigen. The antigen used in their study was a delipidised cercarial extract fractionated on a G-200 column. A sensitivity of 81% was recorded in a group of S. mansoni infected patients. No cross reactions occurred in healthy individuals, but quite a high proportion of patients with other parasitic infections cross reacted. Similar results were reported by Fesenfeld & Parrott (1977) using the same assay procedure. Improved specificity was reported by Weiss et al. (1978) using a radical ergoabsorbent assay (RAST) for IgE detection. Specific IgE antibody was detected in 82% and 72% of patients infected with S. haematobium and S. mansoni respectively, 99% of controls with a variety of other infections were negative. When the same diagnostic titre was applied to the IFAT using sectioned adult worm antigen, the sensitivity of that test was only 38%.

Although the most recently developed immunodiagnostic procedure the enzyme immunoassay (EIA) or enzyme linked immunosorbent assay (ELISA) has already been used in a large number of parasitic infections (Voller et al. 1976). Similar in principle to the IFA the test uses soluble antigens passively adsorbed on to an inert carrier and an enzyme labelled antiglobulin conjugate instead of a fluorescent dye. Its first use in schistosomiasis was described by Ferreira et al. (1974) using particulate adult worm antigen fixed on to microscope slides and a peroxidase enzyme antiglobulin conjugate. Later studies by Huld et al. (1975) and Bout et al. (1976) used what is now regarded as the conventional system, polystyrene tubes or plates with soluble antigen adsorbed on to the surface. In both studies a good distinction was observed between schistosome infected patients and control patients with other helminth infections. Bout et al. (1976) reported the specificity of the test to be 100%.

The test was reported to be less satisfactory when first used in an epidemiological survey in Ethiopia. Polderman & Deelder (1977) found that ELISA did not accurately reflect infection status in several communities with different levels of endemic S. mansoni infection. A high proportion of both false positive and false negative reactions were observed. Their study confirmed the results obtained by Buck et al. (1964) on a similar community but using different techniques.

Before looking at some of the more recent developments in the

Table 1. A summary of some comparative studies on immunodiagnostic tests for schistosomiasis

Reference	Survey	Tests	Sensitivity	Specificity
Buck <u>et al.</u> , 1964	Endemic <u>S. mansoni</u> Ethiopia	Skin test	58%	52%
		CF	71%	38%
		Slide flocculation	45%	66%
		IFAT	79%	17%
Warren & Domingo, 1970	Endemic <u>S. mansoni</u> St. Lucia Controls St. Vincent	CF	44%	86%
		CL	44%	95%
		IF (sensitive)	74%	92%
		IF (specific)	67%	99%
Allain <u>et al.</u> , 1972	Centre for Disease Control screening	BF	57%	38%
		CL	68%	
		CF	48%	
		IFAT	57%	Not determined
		IHA	47%	
Warren <u>et al.</u> , 1973	Endemic <u>S. mansoni</u> St. Lucia Controls St. Vincent	ID (immed)	89-95%	74%
		ID (delayed)	66%	98%
		CF	30-60%	67%
		IFAT	60-80%	89%
		CL	20-50%	90%
Umaly <u>et al.</u> , 1974	<u>S. mansoni</u> infected patients Non-tropical controls	CF	50%	94%
		IHA	85%	100%
		CHR	100%	98%
		IFAT	100%	84%
Polderman & Deelder, 1977	Endemic <u>S. mansoni</u> Ethiopia	ELISA	83%	59%
		DASS	86%	49%
		IFAT	96%	14%
		IHA	82%	37%

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immunodiagnostic field it is perhaps worth attempting to put into perspective what has already been covered, and to try and assess the merits of the different procedures which appear as a diverse and often confusing array of techniques. Some of the most valuable studies in this context are those which have compared the diagnostic performance of a group of immunodiagnostic tests with the same well defined group of patients in a community. A summary of some of these studies are shown in table 1.

In the preceding section an attempt was made to provide some insight into the historical background of the development of immunodiagnostic procedures for schistosomiasis and their epidemiological application, and to examine whether the criticism of this area of schistosomiasis research was entirely justified. Clearly as the summary in table 1 shows, the vast majority of immunodiagnostic procedures fall far short of the listed expectations. Levels of sensitivity and specificity are very variable both between and within tests. There is a complex array of antigens used in the tests and most of these are poorly standardised. It is most probably the variation in the types of antigen used in a test that accounts for the wide disparity in the performance of a particular test in different surveys. With few exceptions the tests are capable of providing only qualitative epidemiological information, and only then when levels of sensitivity and specificity are acceptable. None of the early studies demonstrated any association between egg counts, at present the most valid indicator of infection load, and immunological measurement. With the exception of the COPT no test provided an accurate assessment of the effects of treatment.

The disillusion in the viability of antibody based tests, reflected in the disparaging comment by Davis (1974) undoubtedly spurred on attempts to develop procedures for detecting specific circulating antigens. It was assumed that circulating antigens would not only be very much more specific, but would also relate to the presence of an active infection, to worm burden, and provide a more rapid and accurate assessment of effective treatment.

While schistosome derived antigens have been frequently found in the serum and urine of infected animals by a variety of immunological techniques (Berggren & Weller, 1967; Gold et al., 1969; Deelder et al., 1976), studies in man have proved somewhat less fruitful. There

45%
14%
37%

86%
96%
82%

DASS
IFAT
IHA

Ethiopia

1977

have however been some results to suggest that schistosome antigens may be present in infected individuals. Most of the early work in this field derived from studies on circulating immune complexes, a high proportion of which have been demonstrated in schistosome infected patients (Smith et al., 1975). Attempts were then made to split the immune complexes to enable the individual antigen-antibody components to be detected. Phillips & Draper (1975) described an acidification technique to split the immune complexes and then detected the free antigen and antibody by precipitation in a gel medium following double counter current immunoelectrophoresis (DCIE). Although this method was reported to be very sensitive detecting circulating worm antigens in a high proportion of infected individuals, later reports (Draper, 1976) suggested that false positive reactions may have occurred in the test possibly because of protein isoelectric-precipitation in the gel through the effects of the strong acid. Madwar & Voller (1975, 1977) also demonstrated the presence of circulating schistosome antigens and antibodies by DCIE, without prior acidification of the sera, in 47% and 69% respectively of schistosome infected patients.

Some of the more recent studies on the detection of circulating antigens in human patients have centred on the identification of specific, well characterised antigens, isolated in the earlier animal studies. Two such antigens were of worm origin, polysaccharide in nature but with different charge properties. The earliest of these was identified by Beggren & Weller (1967) in infected animal sera. Later studies by Gold et al. (1969) demonstrated a correlation between the concentration of this antigen in serum or urine and worm burden. Studies by Nash et al. (1974) and von Lichtenberg et al. (1974) characterised the antigen as a high molecular weight polysaccharide located in the schistosome gut. From its properties of migration in an electric field Deelder et al. (1976) termed it the circulating anodic antigen (CAA). This was to distinguish it from another antigen they identified which migrated in the opposite direction and was termed the cathodic circulating antigen. Although CAA could be detected in infected animal sera, Deelder and Eveleigh (1978) were unable to demonstrate its presence in sera from infected humans, despite using a highly sensitive IHA with high titre monospecific antisera, capable of detecting 20 ng/ml of antigen. Even when the sera were concentrated ten times, and when precipitating immune complexes were dissociated no circulating CAA could be detected.

This led the authors to conclude "as the sensitivity of the IHA was good, and control sera failed to show false positive results, the question arises whether detection of CAA in human sera will ever be possible".

Evidence for the presence of the second polysaccharide antigen, CCA in human sera is more encouraging. Deelder & Eveleigh (1978) suggested that CCA was identical to an antigen described somewhat earlier by Carlier et al. (1975), called 'M' antigen. This antigen was identified, by gel diffusion, in the urine of Brazilian patients infected with S. mansoni (Carlier et al., 1975) and in milk from infected mothers (Santoro et al., 1977). The presence of the antigen was also observed to show some association with the intensity of infection.

Recently Santoro et al. (1978) have described the presence of both total circulating schistosome antigen (CSA) and a specific fraction '4' antigen in the sera of S. mansoni infected patients. The fraction '4' antigen, specific for the genus, was present in 61% of infected patients when assayed by the radioimmunoprecipitation - PEG (RIPEGA) test. A correlation between the level of total CSA, fraction '4' antigen and egg count was reported. Since polyethylene glycol (PEG) is commonly used as a method for precipitating complexed immunoglobulins, this assay is as likely to measure the presence of 'preformed' complexes in the serum as 'free' circulating antigen, a point only briefly mentioned by the authors.

As these studies have illustrated there is good evidence to suggest the presence of circulating antigens in schistosome infected patients, with some antigen based systems emerging as potentially useful for epidemiological monitoring of human infection. However, there have been no comparative studies on antigen tests and to date little independent confirmation of the results obtained by different laboratories. While the detection of circulating antigen may in time prove as valuable as anticipated, there have since been some important new developments in the field of antibody assays.

Prominent among these developments is the work by Pelley et al. (1977) on the isolation of a stage and species specific egg antigen (MSA₁) and its incorporation into a highly sensitive RIA for serological

diagnosis of S. mansoni infection. With the assay a number of sera were analysed from two well defined patient populations in St. Lucia and Machakos, Kenya. The assay exhibited good sensitivity detecting antibodies in 100% of infected children and adults in Machakos, a highly endemic area, and in 82% and 98% of children and adults in St. Lucia, a hypoendemic area characterised by low infection levels. The assay also showed exceptional specificity, no cross reactions occurred with the St. Vincent control sera, and in its quantitative form the assay was able to distinguish between the species of schistosomes. The authors report MSA₁ to have a cross reactivity with S. haematobium and S. japonicum of less than 1%.

The relationship between anti-MSA₁ levels and egg counts, in the quantitative assay, was also studied and a significant correlation observed. The assay was therefore capable of providing a measure of infection load in a community. A later study by Long *et al.* (1979) demonstrated that serum eluted from filter paper blood spots could be used in the assay, an important development for epidemiological screening. The results of this study represent an important breakthrough in antibody assays, demonstrating that with the appropriate antigen and assay system highly sensitive, specific and quantitative information can be obtained for S. mansoni infections in communities.

Two other studies also serve to illustrate important developments. Most of these studies are based on the use of 'defined circulating antigens' as the source of antigen in the assay. Nash (1978) reported the use of the gut associated polysaccharide antigen, referred to earlier as CAA, in an IFA test. Fixed paraffin worm sections enabled the removal of antigenic substances other than proteoglycans, so that the specific antigenic region of the gut epithelium could be easily identified. Specific IgG and IgM fluorescent labelled anti-globulin conjugates were used in the test, yielding positivity rates of 86% and 100% respectively in patients infected with S. mansoni. False positive reactions in patients infected with other helminths infections were low, at a level of about 3%.

Kelso & Weller (1978) also described the use of this antigen in a serodiagnostic assay. They obtained a soluble extract of CAA and absorbed it on to microtitre plates in an indirect ELISA. All the S. mansoni infected Brazilian patients tested were found positive in

the assay, and all but one of the control sera, from a wide range of other helminth infections were negative. A positive correlation was also observed in children between their faecal egg output and the level of circulating anti-CAA antibody.

As this chapter has illustrated the immunodiagnosis of schistosomiasis has had a very chequered past. From the start those working in the field have always had to contend with one simple fact, explicitly expressed by Kagan & Pellegrino (1961) in their review, "in judging the value of immunological methods it should be borne in mind that parasitological demonstration of infection is a definite diagnosis, whereas an immunological diagnosis is based on indirect evidence (antibodies formed against the parasite)."

Until the middle of this decade immunological methods made no real impact and did little to contribute to our understanding of important epidemiological parameters in schistosomiasis. This was mainly due to the tests failing to fulfil the two basic requirements of a diagnostic procedure, acceptable sensitivity, to be able to detect infected individuals and specificity, to discriminate between different infections. This consistent failing in almost all the techniques adopted, undoubtedly led to much of the harsh though justified criticism expressed about immunodiagnosis, a subject once facetiously termed "pseudoscience" (Fife, 1971). Since 1975, however, significant progress has been made resulting from a better understanding of the nature of schistosome antigens and the use of sensitive serological techniques. The work of Pelley *et al.* (1977) and Kelsoe & Weller (1978) serves to illustrate this well.

Further developments in this field are still needed. The quality of immunodiagnostic procedures needs improvement so that they can be used as standard recognised procedures. The value of antibody measurements in communities infected with schistosomiasis also needs greater assessment, both in terms of conventional epidemiology, and in attempts to identify groups of patients with different immunological responses. The aim of this thesis was to concentrate on two of these aspects. Firstly to work towards the development and standardisation of the ELISA as a serodiagnostic procedure for *S. mansoni* infection, and then to use it as a tool for studying the role of serology in a range of epidemiological situations.

CHAPTER II

MATERIALS AND METHODS

1. Maintenance of the parasite and isolation of worms and eggs for antigen preparation

Several animals have been used for the laboratory maintenance of S. mansoni, but amongst the most useful because of their high susceptibility to infection are the hamster and mouse. In both these rodents infections become patent some 6-8 weeks after exposure to cercariae with large numbers of eggs becoming lodged in both the gut mucosa and liver. In the liver the eggs give rise to inflammatory reactions which lead to granuloma formation (Warren, 1975).

The mouse provides a particularly convenient laboratory host for isolating large quantities of adult schistosome worms and eggs needed for the preparation of antigens. In this study mice were infected with a Puerto Rican strain of S. mansoni maintained at Winches Farm field station by laboratory passage. All the initial infections were performed at the Farm. Batches of 40 mice were infected at a time, percutaneously, with between 200-250 cercariae, collected after shedding from colonies of the intermediate snail host Biomphalaria glabrata. Cercariae were also collected and concentrated by sedimentation for the preparation of cercarial antigen extracts.

a) Isolation of adult worms from the mesenteries

Eight weeks after infection the mice were anaesthetised by an intraperitoneal injection of 0.25 ml of pentobarbitone/heparin solution (Sagatal) which causes the worms to shift from the mesenteries to the liver. To isolate the worms from the veins the perfusion procedure described by Smithers & Terry (1965) was adopted.

The mice were dissected ventrally to expose the abdominal and pleural cavities, and pegged on to a cork board positioned vertically over a 180 μ m sieve. The hepatic vein was severed and 20 ml of perfusion fluid (1.5% tri-sodium citrate, 0.8% sodium chloride) pumped through the left ventricle with a 21G 1½" needle, and the worms

collected on the sieve. The guts and livers were then removed and separated. The guts were thoroughly washed with phosphate buffered saline (PBS), slit open and cleaned. The livers were crushed between two sheets of plate glass and any additional worms removed. Livers and guts were then placed in separate, covered beakers and kept at 4°C until the following day. Worms collected on the sieve were washed off into a beaker and pooled with those obtained from the crushed livers. They were then washed several times in fresh PBS and adjusted to 1-2 ml packed worms per 10 ml PBS for antigen extraction.

b) Isolation of eggs from gut and liver tissue

Viable ova were extracted from the liver and guts following a method described by Smithers, (1960) with some modification. The washed livers and guts were chopped into pieces with scissors and then small quantities homogenised with some PBS in a MSE bench homogeniser at its highest speed. To the resultant soupy suspension of homogenised guts and livers 0.5 gm of trypsin was added and thoroughly mixed. The suspensions were incubated at 37°C for 2-3 hr in covered beakers.

After incubation the suspensions were re-homogenised and then passed through a 300 μ m and 180 μ m sieve, aided by stirring with a glass rod and the addition of PBS. 15.0 ml of the sieved solution was mixed with an equal volume of PBS in a universal tube and centrifuged at 400 g for 2-3 min. The top layer of liquid was removed by aspiration with a suction pump to within an inch of the bottom of the tube. The sediment was then resuspended in fresh PBS and centrifuged again at 400 g. The cycle was repeated three or four times more until the suspension was reasonably clean. A pool was made of all the sedimented eggs and the suspension poured through the 300 μ m sieve over a large petri dish. By carefully tilting the petri dish any remaining suspended tissue could be removed leaving clean eggs on the base of the dish. The settled eggs were resuspended in PBS, washed and centrifuged at 800 g and the PBS carefully removed and replaced with a fresh solution, 10 ml per 1.0 ml of packed eggs in preparation for antigen extraction. If antigen was not prepared within a few days after extraction the eggs were stored frozen at -60°C.

2. Preparation of schistosome antigens

There is a widely held view that a serological test is only as good as the antigens used in the test, and certainly it has been the lack of specific and well characterized antigens that has limited the use of serological techniques for serodiagnosis of helminth infections (W.H.O., 1975). Mention has already been made of the wide range of antigen preparations that have been used in serological and skin tests for schistosomiasis with varying degrees of success. With the exception of those tests which use the whole parasite as a source of antigen, most intradermal and serological tests depend on the use of soluble extracts prepared from cercariae, eggs and worms. Early preparations were based on homogenisation of the parasite material in buffered saline, which was used either as a crude particulate suspension or centrifuged to yield soluble antigens. These preparations contained highly complex mixtures of antigens as revealed by immunodiffusion studies (Sadun, 1965; Capron, 1965) which were poorly standardised and produced extensive cross reactions in immunodiagnostic tests. Several attempts were made to fractionate the antigens to improve their purity, in particular the adult worm preparations which tended to be contaminated with host protein. Isoelectroprecipitation was adopted by Melcher, (1943) to isolate an acid soluble and insoluble fraction from lyophilised adult worms as a method of purification. Another approach to fractionation was delipidisation of worms and cercariae using alcohol extraction, a technique developed by Chaffee (1954). Although these methods helped to improve the specificity of some immunodiagnostic methods the level of cross reactivity, in particular to other helminth infections, was still unacceptably high.

However, with the recent developments in the field of antigen purification, most notably that of Senft (1975) and Hamburger (1976) coupled with the development of new serodiagnostic assays such as Enzyme Linked Immunosorbent Assay (ELISA), Defined Antigen Substrate Spheres (DASS) and Thin Layer Immunoassay (TIA) which can utilise very small quantities of antigen, there has been a marked improvement in the quality of immunodiagnosis of schistosomiasis.

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from cercariae, worms and eggs and divided simply into two broad categories of somatic extracts, prepared from parasite body tissue, and exo-antigens prepared from parasite excretory secretory products. Most early work on schistosome antigens has been based on somatic antigen preparations, but there are now increasing attempts to isolate metabolic antigens which being released from living parasites only, may relate more closely to the presence of an active infection in the host (Sadun, 1976).

The following antigen extracts were prepared:

Somatic Antigen Preparations :

Cercarial homogenate

Worm membrane antigen

Egg homogenate

Exo-antigen Preparations :

Worm excretory secretory antigen

Cercarial homogenate

Suspensions of 1×10^6 of cercariae, collected from infected snails, were sedimented by centrifugation at 10,000 g. The pellet was resuspended in 5.0 ml of cold phosphate buffered saline (PBS), pH 7.6 and aliquots of the suspension ground in a precooled glass hand homogeniser. After thorough grinding the solution was left at 4°C overnight for full extraction. The solution was then centrifuged at 2,500 g for 20 min and stored at -60°C, an aliquot being retained for biochemical analysis.

Worm membrane antigen

Two techniques were used to isolate the outer membrane of the adult worm to produce a soluble extract. The first technique involved a process of freezing and thawing following a method described by Ramalho-Pinto *et al.* (1976) and the second a method of separation using concentrated salt solutions following the basic method of Vannier *et al.* (1974).

a) Isolation by freezing and thawing

Following perfusion isolated worms were washed several times in PBS to remove host protein contaminants and then resuspended in fresh PBS, 1-2 ml of packed worms to 5.0 ml of buffer. The worms were frozen at -60°C and then rapidly thawed in a water bath at 37°C. The worm suspension was again frozen and thawed twice more, before the worms were separated from the supernatant by filtration. The

supernatant was retained and the worms resuspended in a further 5.0 ml of fresh PBS. The cycle of freezing and thawing was repeated, the two supernatants pooled and centrifuged at 800 g. The supernatant was stored at -60°C after an aliquot had been retained for biochemical analysis.

b) Isolation by concentrated salt solutions (KCl)

After perfusion 1-2 ml of washed packed worms were suspended in 10 ml of 3M KCl in Hanks buffered salt solution pH 7.4 and stirred for 12 hr at 4°C . The supernatant was separated by filtration and centrifuged at 800 g. To remove excess salt the supernatant was passed down a 15 cm glass column packed with Sephadex G-25 and equilibrated with PBS, with volumes of 1-2 mls of antigen being applied to the column at a time. The solution was then concentrated to a final volume of 5-10 ml by ultrafiltration (Amicon, PM10) before storage at -60°C . As with previous preparations an aliquot of antigen was retained for biochemical analysis.

Egg homogenate (SEA)

A soluble egg antigen was prepared by homogenisation following the basic method of Boros & Warren (1970). Viable ova isolated from infected mouse livers and guts were washed in PBS and allowed to sediment. 1.0 ml of packed ova were suspended in 10 ml of PBS, and an aliquot of the suspension ground in a precooled hand homogeniser. To check that the ova were thoroughly crushed a small quantity of the suspension was examined under the microscope, with a few drops of Lugols iodine being added to the suspension to more readily identify whole ova.

After overnight extraction at 4°C the suspension was centrifuged at 2,500 g and the supernatant stored at -60°C .

Excretory secretory worm antigen

Complex culture mediums have been used for the isolation of metabolic antigens, but this can result in the antigens becoming contaminated with products of the culture medium. Since maximum release of schistosome excretory secretory material occurs within a few hours after isolation from the mammalian host, worms can be adequately incubated in saline yielding antigens which can then be used in diagnostic assays. A method described by Deelder, (1976) for the incubation of worms in isotonic saline was used. After perfusion worms were washed three times in warm (37°C) isotonic saline and then transferred immediately to a sterile culture flask containing 10 ml

Table 2. Biochemical analysis of crude and ultracentrifuged *S. mansoni* cercarial, worm and egg antigens

Basic preparation	Average protein conc. ug/ml		Average carbohydrate conc. ug/ml
	crude	uc*	
Cercarial homog.	250-500	125	100
Adult worm membrane			
F/T	500-1000	300-500	50
KCl	500-1000	300-500	50
Egg homog.	1000-2000	300-500	150

* uc - ultracentrifuged at 100,000 g.

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of isotonic saline. The culture flask was then placed in an incubator at 37°C for 2-3 hr, which is the time of maximum release of gut contents with minimum worm death. After incubation the suspension was filtered and the antigen stored at -60°C. Biochemical analysis was carried out on an aliquot of the antigen.

Ultracentrifugation

Although low speed centrifugation, 800 g and 2,500 g used in this study, is sufficient to remove large particulate material such as intact cells and tissue debris, centrifugation at very much higher speeds, 100,000 g, is essential to remove all cellular organelles and obtain soluble proteins (Conn & Stump, 1976). Ultracentrifugation was used therefore to produce completely soluble protein antigens for use in ELISA.

The frozen crude antigen preparations were thawed and spun in an MSE ultracentrifuge at 100,000 g for 1-2 hr at 4°C. 250 ul aliquots of the supernatant were then stored at -60°C after the antigen had been biochemically analysed.

As the results in table 2 show ultracentrifugation removes between 50 and 75% of the protein in a crude preparation and thus offers a simple method for removing some potentially cross reactive antigen components.

Biochemical analysis

Biochemical analysis of the antigen preparations for total protein and carbohydrate was used to obtain basic information on the organic composition of the different antigen preparations and as a method of standardisation. The limitations of this approach must however be stressed in that protein and carbohydrate concentration does not necessarily reflect the 'antigenic reactivity' of a particular preparation. Until more refined immunological methods are produced, possibly typing with monospecific antisera, biochemical characterisation provides a reasonably convenient method.

Two techniques were selected for protein estimations because of their sensitivity in the low protein concentration range, the Folin

Lowry assay and a recently developed assay from Biorad Laboratories.

Folin Lowry Protein Assay (sensitivity 0.01-0.2 mg/ml)

This protein assay is a colorimetric method which relies on the presence of the amino acids tyrosine and tryptophan in a protein solution. The method developed by Lowry (1951) was used.

The following solutions were prepared:

- A) 2% Na_2CO_3 in 0.1M NaOH
- B) 0.5% $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 1% sodium tartrate
- C) 50 ml of solution A mixed with 1.0 ml solution B
- D) Folin - Ciocalteu phenol reagent (BDH Chemicals)
- E) Bovine serum albumin protein standard (BSA - Sigma)

From a 30% stock solution of BSA the following concentrations were prepared, 500, 250, 100, 50, 25 $\mu\text{g}/\text{ml}$ protein in distilled water. 1.0 ml of each standard was pipetted into a test tube, and 1.0 ml of distilled water into a separate tube as a blank. An aliquot of the antigen sample to be measured was diluted to a final volume of 1.0 ml and pipetted into a test tube. To each tube 5.0 ml of reagent C was added, the contents thoroughly mixed, and left to stand at room temperature for 10 min. 0.50 ml of reagent D was then added to each tube and again they were thoroughly mixed. After standing at room temperature for 30 min the samples were in turn transferred to a cuvette and the absorbance at 625 nm recorded, the distilled water blank being used to set the absorbance scale to zero. A calibration curve was plotted from the absorbance readings of the BSA protein standards and the concentration of the unknown antigen samples read from the graph.

Biorad Protein Assay Kit (Sensitivity 0.01-0.2 mg/ml)

This method of protein estimation, devised by Bradford (1976) is based on the binding of a specific dye, Comassie blue G-250 to protein. The binding of the dye to the protein causes a shift in the absorption maximum of the dye from 465 nm to 595 nm. The increase in absorption at 595 nm following binding can then be used to measure the concentration of protein in a solution. Both the concentrated dye reagent and albumin protein standard are supplied in kit form (Biorad

500-0001).

The dye reagent was diluted five times and filtered to form a working solution. The protein standard was reconstituted to a final concentration of 1.4 mg/ml in distilled water and concentrations from 500-25 μ g/ml prepared. 0.1 ml of each standard, and 0.1 ml of distilled water as control were pipetted into tubes. The antigen solutions for testing were diluted to 1/4 with distilled water and a 0.1 ml aliquot pipetted into a tube. To each tube 5.0 ml of the dye reagent was added and the contents thoroughly mixed. After standing for 5 min at room temperature the samples were measured photometrically at 595 nm. The distilled water blank was used to set the absorbance scale to zero. A calibration curve was plotted with the OD values of the protein standards and the unknown concentration of the antigens read from the graph.

To determine the carbohydrate content of the antigen preparations the anthrone method was used following a procedure described by Mokrasch, (1954).

The stock reagent was prepared as follows. 136 ml of concentrated sulphuric acid (17M) was added to 64 ml of distilled water contained in a beaker and placed in an ice bath to dissipate the heat of reaction. This work was carried out in a fume cupboard. To this solution 2 g thiourea and 0.1 g of anthrone reagent were added and mixed thoroughly. This solution could be stored at 4°C for a period of up to 1 month.

Prior to starting the assay a waterbath was brought to a temperature of 80-90°C. From a stock solution of sucrose the following concentrations were prepared: 500, 250, 150, 100 and 50 μ g/ml. In a chilled water bath 5.0 ml of anthrone reagent was pipetted into the required number of tubes. 0.5 ml of standard sucrose solutions and antigens under test were then carefully layered on to the anthrone solution. Each tube was carefully mixed and left in the water bath to dissipate the heat of reaction. The tubes were then immersed in the water bath at 90°C for 15 min. After cooling the solutions were transferred in turn to a cuvette and the absorbance at 625 nm recorded. Values obtained for the sucrose standards were used to plot a calibration curve and the unknown concentrations of the antigens read from the graph.

3. Indirect Enzyme Linked Immunosorbent Assay

Some of the most successful assays for the measurement of antibodies in serum are the solid phase immunoassays. These systems depend on the capacity of antigens to bind to inert carrier surfaces forming an immunoadsorbent for the subsequent attachment of antibody. The amount of specific antibody present is then measured by reacting it with a labelled antiglobulin.

Fluorescent labels have been used extensively in indirect assays for the measurement of antibodies to a large number of viral, bacterial and protozoal infections. In these cases layers of infected cells adsorbed on to the surface of microscope slides act as antigen. In some helminth infections soluble protein antigens have been covalently linked to agarose beads (Deelder, 1975a) forming the basis of an assay called the Defined Antigen Substrate Spheres, DASS. In both systems the reactions are usually visualised by ultraviolet microscopy and quantitation of the antibody based on titrating the serum on a two or four fold dilution series to a selected end point. The subjective assessment of the results is one of the inherent disadvantages of the assay and recently there have been attempts to improve quantitation by fluorometric measurements (Deelder, 1978; Manawadu & Voller, 1978).

The second major development in the field of indirect labelled assays was the radioimmunoassay, in which the antisppecies globulin is bound to a radioactive isotope such as I^{125} . Soluble antigen is adsorbed on to an inert carrier, such as a plastic tube, and after antibody has bound, the conjugate is added to form a complex. The amount of radioactivity in the complex then provides a sensitive measure of the concentration of antibody in the serum. Although radioimmunoassay provides an accurate quantitative measure of the amount of antibody present in serum it has certain drawbacks, in particular the hazards associated with working with isotopes. For this reason and the high operation costs of the system, it has not had wide application in the field of parasitology.

Somewhat surprisingly the solid phase enzyme immunoassays (EIA/ELISA) were the last of the labelled techniques to be used in indirect antibody assays. Although enzyme labelled antiglobulin

Figure 2 . The indirect ELISA for measuring antibody

1. ANTIGEN ADSORBED TO PLATE

WASH

2. ADD SERUM ANY SPECIFIC
ANTIBODY ATTACHES TO ANTIGEN

WASH

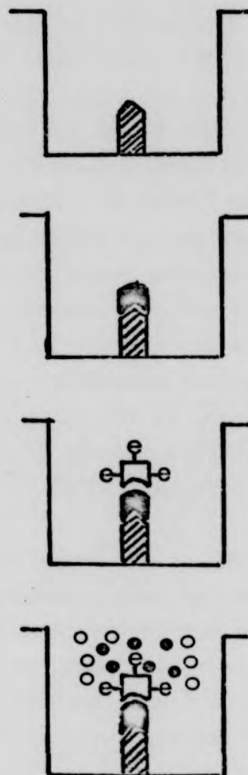
3. ADD ENZYME LABELLED ANTI-
GLOBULIN WHICH ATTACHES TO
ANTIBODY

WASH

4. ADD SUBSTRATE

AMOUNT HYDROLYSED = AMOUNT OF ANTIBODY PRESENT

(Reproduced from Voller et al, 1976, Bull W.H.O. 53,55-65)



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conjugates have been used for several years for the identification and localisation of immune reactants in histological preparations (Avrameas, 1969; Nakane, 1966), it was not until the early 1970's through the independent work of Van Weeman and Schuurs, (1971, 1972) and Engvall and Perlmann (1972) that their potential for use in solid phase immunoassay was recognised.

The principle of the indirect Enzyme Linked Immunosorbent Assay (ELISA) for the measurement of specific antibody is illustrated in figure 2, and is similar in principle to both solid phase indirect immunofluorescent antibody tests and radioimmunoassay. Basically the system involves the passive adsorption of soluble antigen at alkaline pH to the surface of a polystyrene tube or plate. After incubation the surface is washed to remove unbound antigen. Diluted serum is then added and incubated for several hours. The plate is again washed to remove excess non complexed antibody and to prevent non-specific binding. Diluted enzyme labelled anti-species globulin conjugate is then added and the tube or plate incubated for a further few hours. After incubation the plate or tube is again washed to remove excess conjugate and the specific enzyme substrate added. The enzyme and substrate are chosen so that the product of enzyme substrate breakdown is coloured and this can then be assayed photometrically at an appropriate wavelength. The intensity of the coloured end product provides a measure of the concentration of the specific antibody in the serum under test.

The application of enzyme labelled antibody techniques to parasitic infections has resulted largely from the work of Voller, (1976a). One of the earliest applications of the technique was for the serodiagnosis of malaria infections (Voller *et al.*, 1974). In this study the microplate technique was first used with soluble malaria antigen coated on to the wells of a microtitre plate instead of polystyrene tubes as used in the initial development of the assay. This development made the technique particularly well suited to large scale epidemiological surveys. A serological survey for antibodies to *T. cruzi* infection in Brazil (Voller *et al.*, 1975a) showed another valuable application of the technique. Comparisons with the standard indirect fluorescent technique showed excellent matching between the tests of the order of 98%. Other applications of the technique for protozoal infections include its use for the serodiagnosis of African

trypanosomiasis (Voller et al., 1975b) leishmaniasis (Hommel, 1976), Amoebiasis (Bos et al. 1975) and Toxoplasmosis (Voller et al., 1976b; Bout et al., 1976).

The indirect antibody technique has also found a use in virological diagnosis being used for the detection of antibodies in measles, cytomegalovirus infections and for Rubella using specific IgM detection (Voller et al., 1976c). It has also proved a useful technique for the diagnosis of fungal infections, which can be difficult to diagnose by conventional microbiological techniques. Warren et al. (1977) demonstrated its use for Candidiasis and McLaren et al. (1978) for a range of fungal mycoses.

It is, however, for serodiagnosis of helminth infections that enzyme immunoassays could make greatest impact. Since the assay demands only minute quantities of antigen, highly purified antigens can be used in the test or crude antigen preparations can be diluted to very low concentrations thus removing large amounts of potentially cross reactive antigen components, which have previously handicapped the use of serological methods for serodiagnosis of helminth infections. Bout et al. (1976) showed the value of the assay for detecting antibodies in patients with hydatid disease using both whole cyst fluid antigen and the highly purified E. granulosus specific fraction five antigen. From initial experimental work on the use of the assay for antibody studies in trichinosis in pigs, the technique has now been incorporated into a programme for routine screening of pigs for T. spiralis infection (Ruitenbergh et al., 1975), and by Engvall & Lungstrom, (1975) for human trichinosis. Its use in filarial infections has to date been fairly limited owing to the problems of antigen cross reactivity between the different filarial worms. Bartlett & Bidwell (1975) demonstrated that the technique could be used for measuring antibodies in patients with Onchocerciasis, but a heterologous species O. gutturosa from pigs had to be used as a source of antigen, since the homologous antigen gave many cross reactions with other related nematode infections. Of the nematode infections difficult to diagnose by conventional parasitological techniques toxocariasis has been successfully diagnosed serologically using ELISA and specific antigens from cultured T. canis larvae (de Savigny, 1979). Sero-diagnosis of schistosomiasis has also been successfully achieved using ELISA. Huldt et al., (1975a) applied the technique to a small study of

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S. mansoni infected patients and uninfected controls, as did Bout et al. (1976).

Comparisons have also been made between enzyme immunoassays and other indirect labelled techniques, in particular radioimmunoassay. Voller et al. (1977) carried out a large scale comparative study of isotopic and enzyme immunoassays for parasitic diseases using the indirect antibody technique. They found excellent agreement between antibodies measured by both assays in Chagas disease, African trypanosomiasis, amoebiasis, malaria and schistosomiasis. Schinski et al. (1976) also found good correlation between RIA and ELISA when used on serum from patients with schistosomiasis.

The successful application of the technique to such a wide range of infections and its good qualitative and quantitative agreement with the widely accepted radioimmunoassay, plus the advantages of stable antiglobulin conjugates and the absence of health hazards associated with isotopes was recently reviewed (Lancet, 1976) in a paper interestingly entitled 'ELISA: a replacement for RIA?'. This optimistic view was not however shared by all workers and criticism has been levied at the lack of standardisation, in particular of the enzyme conjugates, and precision of the technique (Landon, 1977).

In addition, therefore, to studies on the application of the technique to infectious diseases an increasing amount of work is now being carried out on improving the methodology of the assay through detailed studies on different test parameters. Bullock & Walls, (1978) have recently carried out studies to evaluate some parameters of the assay, such as type of carrier surface for antigen adsorption, reproducibility of antibody titre and most suitable enzyme labels and substrates using their toxoplasma antibody system.

In developing this assay for serodiagnosis of schistosomiasis a number of similar experiments were undertaken with the object of producing a reliable 'standardised' test for use in a range of epidemiological studies. The initial phase of the work involved the standardisation of test reagents and basic test parameters followed by more detailed quality control studies.

a) Preparation and standardisation of peroxidase labelled
antiglobulin conjugates

Although there are several enzymes which have been linked to antibodies the two which have been used most extensively are horseradish peroxidase and alkaline phosphatase. Both enzymes satisfy many of the criteria essential for the production of a good conjugate. These include, isolation of the enzyme in a relatively pure form, a high turnover number (i.e. one molecule of enzyme can break down several molecules of substrate) and an end product of substrate degradation which can be measured photometrically (Schuurs & Van Weeman, 1977).

Several methods exist for coupling these enzymes to antibodies, the main requirement being that when coupled the enzyme and antibody must retain their inherent reactivity as independent molecules. In this study two of the most commonly used methods were selected for conjugating horseradish peroxidase to antihuman gamma globulin, firstly the periodate method and secondly the two step glutaraldehyde method.

The periodate method

This method is based on the coupling of horseradish peroxidase via its carbohydrate moiety to the antibody, the technique developed by Nakane, (1974) was used.

Step 1. Preparation of peroxidase - aldehyde solution

5.0 mg of horseradish peroxidase (Sigma type VI Rz 3.0) was weighed and dissolved in 1.0 ml of 0.3M sodium bicarbonate buffer. To this 0.1 ml of 1% fluorodinitrobenzene in absolute ethanol was added. This addition being carried out in a well ventilated fume cupboard. The mixture was then mixed gently for 1 hr at room temperature (19-22°C). After mixing 1.0 ml of 0.06M sodium periodate was added and the resultant solution mixed for a further 30 min at room temperature. 1.0 ml of 0.16M ethylene glycol was then added and the solution mixed for 1 hr at room temperature. The total solution volume of 3.1 ml was then transferred to a dialysis bag and left to dialyse against a 0.01M carbonate buffer solution pH 9.5 for a day

with several changes of buffer. 0.01% Thimersal (Merthiolate) was then added to the conjugate as a preservative. If not used immediately the solution was stored in the dark at -20°C ; at 4°C it was stable for about 1 month.

Step 2. Preparation of peroxidase labelled antibody

The gamma globulin fraction of the serum was prepared from total antiserum by sodium sulphate precipitation following the method described by Voller *et al.*, (1976d). The concentration of the re-dissolved dialysed precipitate of gamma globulin was determined by spectrophotometry at 280 nm and adjusted to 5.0 mg/ml protein.

5.0 mg of lyophilised protein was added to 3.0 ml of the peroxidase-aldehyde solution and mixed for 2-3 hrs at room temperature. The solution was then transferred to a dialysis sac and dialysed at 4°C against phosphate buffered saline (PBS) overnight. The resultant conjugate was then screened for reactivity using the standardised assay procedure.

The two step glutaraldehyde method

The method developed by Avrameas, (1971) was used, and contrasts with the one step conjugation procedure more commonly used for alkaline phosphatase, in that enzyme and glutaraldehyde are first reacted and then the second protein is coupled after removal of excess glutaraldehyde. This technique is very suitable for horseradish peroxidase because of its low amino group content which does not cause excessive cross linking in the first step.

Step 1. Horseradish peroxidase - glutaraldehyde coupling

10 mg of peroxidase (Sigma type VI Rz 3.0) was dissolved in 0.2 ml of 0.1M phosphate buffer pH 6.8 containing 1.25% glutaraldehyde (Sigma type II). The mixture was left to stand at room temperature for 18 hr. The solution was then filtered through a column containing Sephadex G-25 equilibrated with 0.15M NaCl, and concentrated through an Amicon PM10 ultrafiltration membrane to a final volume of 1.0 ml.

Step 2. Antibody coupling

5 mg of gamma globulin, dissolved in 1.0 ml of 0.15M NaCl was

added to the HRP solution, and a further 0.1 ml of 1.0M carbonate buffer pH 9.5 added. The solution was left to stand for 24 hr at 4°C. 0.1 ml of 0.2M lysine was then added and the mixture left for 2 hr at room temperature. The conjugate was then precipitated with saturated Na_2SO_4 , washed twice with half strength solution and dissolved in a minimal volume of distilled water, and then dialysed exhaustively against PBS. It was then centrifuged at 20,000 g for 20 min, sterilised by the addition of 0.01% merthiolate and stored at 4°C or -20°C long term.

Purification of enzyme conjugates

The composition of the conjugates prepared by either of the above methods will consist of enzyme linked antibody and both free enzyme and unlabelled antibody. The non labelled immune reactant should be removed from the conjugate preparation because it will tend to lower the sensitivity of the assay, and the free enzyme since it will tend to increase non specific background reaction in the assay. Several methods have been used for purification with gel filtration through Sephadex G-100 or G-200 being the most widely accepted. Sephadex G-200 was chosen in this study because of its high resolution.

Preswollen Sephadex G-200 beads were packed under gravity in a 40 cm glass column with a glass fibre bed support. The column was equilibrated with several washes of PBS pH 7.2, and 1.0 ml of 1% blue dextran passed through the column to determine the void volume. After further washing the column was ready for use.

2.0 ml of conjugate was applied to the surface of the G-200 column and filtered through, 2.0 ml fractions being collected and the eluent monitored at 280 nm which measures protein activity and 403 nm for peroxidase activity. Figure 3 shows the elution profile for a conjugate prepared by periodate linking. The fractions which contain activity at both 280 nm and 403 nm were pooled and concentrated by filtration through an Amicon ultrafiltration membrane PM10 with an exclusion limit of molecular weight 10,000. The purified conjugate was then screened to determine its working strength.

Figure 3. Elution profile of a peroxidase labelled antiglobulin conjugate prepared by periodate linkage.

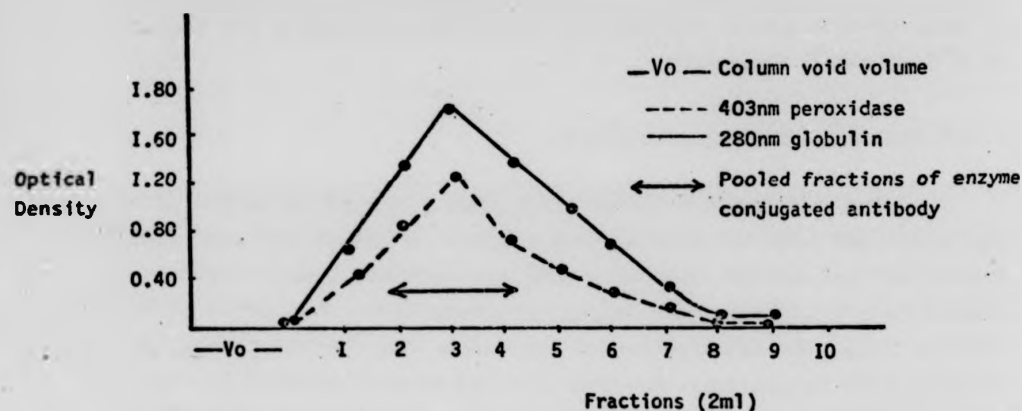
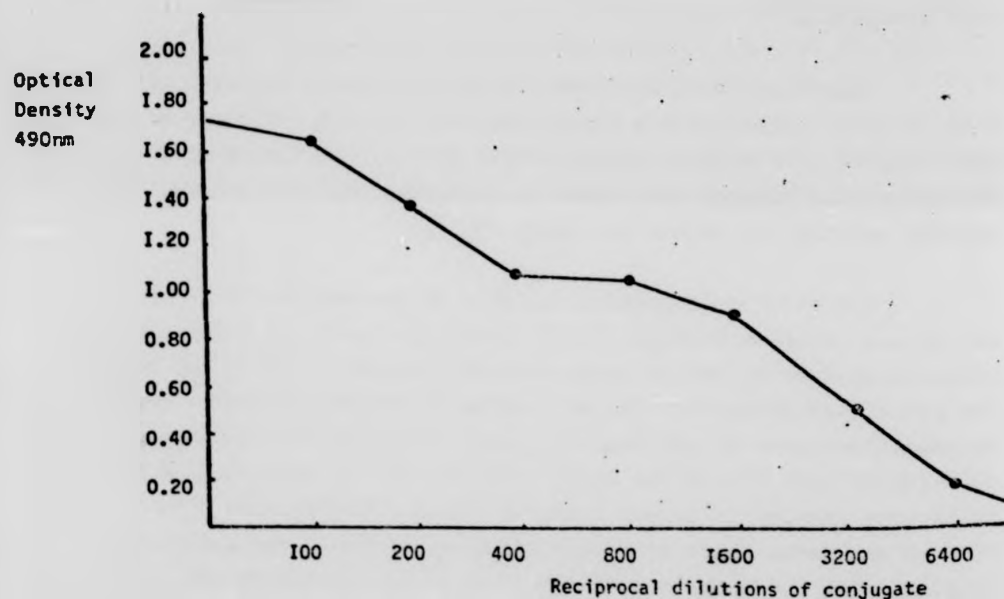


Figure 4. Determination of optimum dilution of enzyme labelled anti-globulin conjugate.



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Determination of conjugate working strength

To determine the optimum dilution of the peroxidase labelled antiglobulin conjugate for use in the assay the method described by Voller et al. (1976) was used with a slight modification.

200 μ l of a standard preparation of human IgG (Behringwerke) diluted to a concentration of 100 ng/ml (Voller et al., 1976e) in coating buffer was added to the wells of a microtitre plate (Dynatech M129A) and left covered in an incubation chamber at room temperature overnight. After washing the plate three times for three minutes in Tween 20 - saline washing solution, 200 μ l of twofold reciprocal dilutions of conjugate in incubation buffer were added to the plate and incubated for three hours. Further washing as above was carried out to remove excess unbound conjugate and then 200 μ l of the specific peroxidase substrate orthophenylene diamine - H_2O_2 was added. The enzyme substrate reaction was allowed to develop for 30 min after which the reaction was stopped by the addition of 25 μ l of 8N H_2SO_4 which inhibits any further breakdown. The coloured end product was then transferred to a microcuvette and the optical density readings from the photometer recorded.

From the graph illustrated in figure 4 the dilution giving an optical density value of approximately 1.0 (Voller et al., 1976e) was selected as the optimum working dilution of the conjugate to be used in the assay. A dilution of about 1/1000 corresponds with this level.

b) Determination of the optimum concentration of antigen and dilution of serum in the test

Preliminary chequer board titrations were carried out to determine the optimum concentration of antigen and dilution of serum to be used in the assay. Standardisation of antigens was based on biochemical analysis for total protein and carbohydrate, and protein concentration was used for determining optimum levels of antigen coating since this group of molecules adhere predominantly to polystyrene surfaces.

To determine optimum antibody dilutions three serum samples were used. A reference positive, prepared by pooling several high

Table 3. Determination of optimum antigen concentration and dilution of serum for use in ELISA. Calibration data for soluble egg antigen at E490/30min/RTtoC

Serum dilution	Antigen concentration $\mu\text{g/ml}$ protein				
	25.00	5.00	2.50	1.00	0.50
1/150	1.32 ^a /0.63 ^b /0.14 ^c	1.33/0.63/0.21	1.29/0.61/0.13	0.97/0.75/0.11	0.62/0.36/0.12
1/300	1.22/0.38/0.07	1.21/0.40/0.11	** 1.16/0.38/0.09	0.97/0.33/0.23	0.49/0.22/0.07
1/600	0.93/0.21/0.08	0.89/0.22/0.05	1.13/0.23/0.11	0.65/0.21/0.05	0.41/0.12/0.05
1/1200	0.78/0.12/0.05	0.73/0.24/0.03	0.69/0.14/0.04	0.45/0.13/0.02	0.29/0.09/0.03

a = pooled strong positive 'reference' serum

b = pooled weak positive control serum

c = pooled negative control serum

** = selected optimum level

titre sera, a weak control positive and a pool of negative sera from uninfected controls.

The basic procedure outlined by Voller et al. (1976e) with regard to incubation times and temperatures was adopted. Dilutions of the stock antigen solution were made in coating buffer, and 200 μ l of each were pipetted into the 96 wells of the microtitre plate. The plate was covered and incubated in a sealed container (incubation chamber) overnight at room temperature. After rinsing the plate with washing solution, 200 μ l of different dilutions of the reference and control sera were added to appropriate wells, the plate covered and incubated at room temperatures for 2 hrs. The plate was again washed, this time three washes each for three minutes before 200 μ l of the enzyme conjugate diluted to its predetermined working strength (see 4) was added to each well. After incubation at room temperature for 3 hrs the plate was washed as above and the specific enzyme substrate added. The reaction rate was stopped after 30 min by the addition of an appropriate inhibitor and the optical density (absorbance) values determined for each sample from spectrophotometric readings at 400-405 nm for phosphatase and 490-492 nm for peroxidase.

Table 3 shows a series of calibration data for soluble egg antigen (SEA). 2.5 μ g/ml protein was selected as the optimum level of antigen for coating the plates as this was the minimum concentration necessary to achieve a condition of 'antigen excess'. As the table shows increasing the concentration of antigen does not increase appreciably the amount of antibody that binds, this applies for both the positive sera. These concentration levels are similar to those obtained by Engvall & Perlmann (1972) who estimated that at concentrations of the order of 2 μ g/ml over 60% of antigen was adsorbed on to the tube, but this percentage decreased as protein concentration was increased.

A serum dilution of 1/300 was selected as the optimum for testing. At this level there was a good distinction between positive and negative controls especially the weak positive which would have been lost at higher dilutions. At lower serum dilutions there is the possibility of obtaining sera in antibody excess, where there is insufficient antigen to bind all the available antibody and this has to be taken into account in selecting optimum dilution levels.

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Both adult worm and cercarial antigens were used at a higher optimum concentration of 5 μ g/ml, with sera at the single dilution of 1/300.

c) Optimisation of reaction endpoint

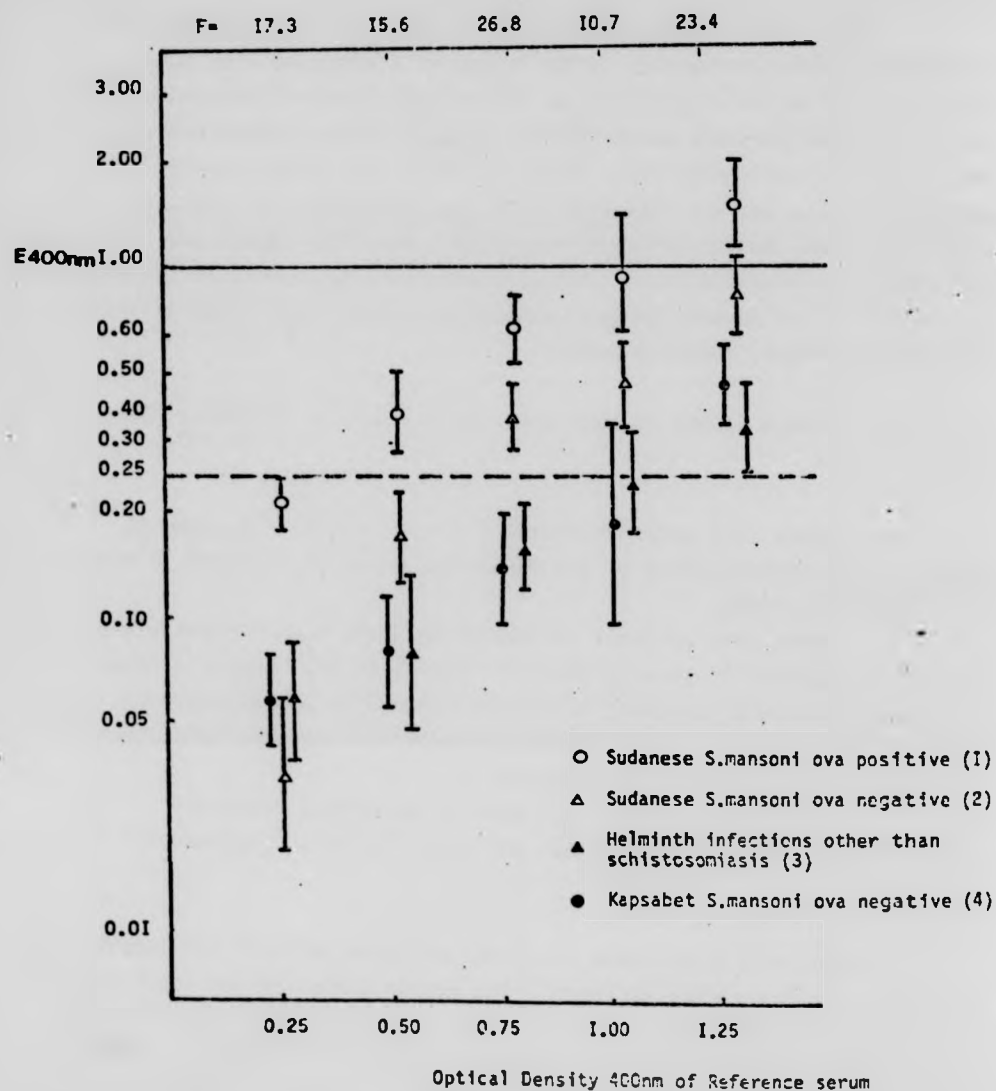
Many workers using ELISA have stopped the reaction rate of enzyme-substrate interaction on the basis of a selected time interval (30 min-1 hr) or in relation to an arbitrarily selected absorbance (OD) of a reference positive serum (Voller *et al.*, 1976e). Reactions based on a selected absorbance value should improve day to day sample reproducibility because this method is less dependent on variables such as ambient temperature and substrate composition which can influence the reaction rate. The approach has been developed further in an attempt to select the most appropriate absorbance level at which to stop the enzyme reaction rate.

A random sample of sera were taken from the following groups listed below:

1. 15 sera from parasitologically proven cases of S. mansoni from a highly endemic area in the Sudan who could be expected to have high antibody levels.
- 2 15 sera from subjects living in the same highly endemic area, but who had parasitologically negative stools at the time of taking blood but who would certainly have been exposed to schistosomiasis.
3. 15 sera from U.K. hospital patients with various helminth infections other than schistosomiasis.
4. 15 sera from people in an area of E. Africa (Kapsabet) where there is no schistosomiasis, but where there is exposure to many other helminth infections.

Replicate ELISA tests of these sera were carried out together with the positive reference serum. The enzyme-substrate reaction was stopped when the absorbance values of the reference serum had reached 0.25, 0.50, 0.75, 1.00, 1.25. At each of these levels the mean and 95% confidence intervals for each group were calculated. Graphical analysis showed the standard deviations to be increasing with reaction development and so the data were transformed into logarithms to make the standard deviations more uniform. To compare differences between the groups variance ratios (F) were calculated at each point. The

Figure 5 . Geometric means of OD levels of different categories of sera read at different set OD values of a reference serum. Vertical bars indicate 95% confidence limits, F= variance ratios calculated at each point, with values of F 4.13 as significant at p 0.01



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point at which there is greatest difference between groups and smallest variation within groups will give the highest value for F i.e. the point where differences are likely to be most significant. Figure 5 shows this level to be when the reference positive serum reached an optical density level of 0.75.

In all subsequent tests replicates of the reference positive serum were included in each plate, and the absorbance of the reference monitored until it reached a level of 0.75 at which point the reaction in all other wells was stopped.

Several methods have been used to express the results of antibody levels measured in an enzyme immunoassay. The easiest and most common method is to express the results in absorbance (optical density) values. This requires setting diagnostic absorbance levels for seropositivity and negativity. An approach which was adopted in this study. A diagnostic absorbance level of 0.25 was selected to differentiate between a positive and negative antibody response. Values of less than and equal to 0.25 were taken to be negative and greater than 0.25 as positive. This was an arbitrarily selected level just above the upper confidence intervals for the two categories of S. mansonii negative sera, and favours test sensitivity at the expense of some specificity.

Absorbance values provide a highly quantitative form of expression, but they are not widely understood as a serological measure as for example an end point titre, and it is possible to get 'clumping' in the high OD range.

Titration by serial dilution to a selected end point, as used in most other serological tests is also a popular form of expression. This method has the advantage of being widely understood as well as being highly quantitative. The large dilution ranges used in ELISA, usually $\times 10$, does however make this an inaccurate method. This approach has been used for measuring increases in antibody levels after rabies immunisation (Thraewhart & Kuwert, 1977) and for influenza screening (Leinikki & Passila, 1976).

The non linear relationship between absorbance and titre makes it difficult to relate the two measures, although a system called the multiple of normal activity (MONA) has been devised. Two other methods

not widely used because they are poorly quantitative are visual expression and positive negative ratios.

d) A summary of the technique developed for routine serological work based on the standardisation of test parameters

Day 1

Preparation of serum and blood samples for testing

Serum samples:

Serum samples were screened at a single optimum dilution of 1/300. This was prepared by thoroughly mixing 5 ul of serum in 1.50 ml of incubation buffer. Care was taken to ensure the tubes were marked with the appropriate serum code. The diluted samples were then covered and kept at 4°C overnight.

Blood eluates:

Using a calibrated punch, discs of dried blood containing the equivalent of 30 ul of whole blood were cut. To each folded disc, placed in a small tube (LP3), 0.75 ml of incubation buffer containing 1% bovine serum albumin (BSA) as stabiliser was added to give a serum dilution of 1/50. The samples were then covered and placed in a refrigerator at 4°C overnight.

A total of 150-200 samples could be prepared for testing at any one time.

Preparation of antigen coated plates with soluble egg antigen

An aliquot of the stock antigen solution (500 µl/ml protein) was diluted 1/200 to the optimum working strength of 2.5 µg/ml in coating buffer. To each well of a microtitre plate (M129A) 200 ul of diluted antigen was pipetted using a variable volume multichannel pipette. The plate was covered and left overnight at room temperature in a sealed container.

Day 2

If blood eluates were to be tested the paper discs from which the blood has been eluted were removed and discarded into a bowl of disinfectant (chlorox). In between removing the paper discs

the applicator was rinsed in water.

Dilutions of 1/300 aliquots of the reference and control sera in incubation buffer were prepared. The sera consisted of a) a pooled positive reference for endpoint determinations, b) a pooled control positive to assess reproducibility of test measurements and c) a pooled negative to check for non specific background reaction.

Test run:

The antigen coated plates were emptied and rinsed twice with washing buffer. The reference and control sera were added to the plate first; six replicates of the reference positive in wells A 1-6, three replicates of the control positive and negative in rows A7-9 and A10-12 respectively, to the remaining wells 200 μ l of diluted serum samples were added. For the blood eluates 180 μ l of incubation buffer was pipetted into each well and then 30 μ l of eluted blood added to give a final dilution of 1/300. In each plate a number of wells containing just PBS (incubation buffer) were left as controls. The sample numbers were then recorded on the assay test sheets and the plate covered and placed in an incubation chamber for 2 hrs at room temperature.

After incubation the plate was emptied and rinsed. It was then flooded with washing solution and left to stand for 3 mins, this was repeated twice more. During the washing cycle the appropriate dilution of enzyme antiglobulin conjugate was prepared in incubation buffer. When the washing cycle was completed 200 μ l of diluted conjugate was pipetted into each well using a multichannel pipette. The plate was again covered and incubated for 3 hrs at room temperature.

Half an hour before the final washing phase the photometer was switched on so that it had time to warm up, and the appropriate interference filter selected, 400-405 nm for phosphatase and 490-492 nm for peroxidase.

After the three hour incubation period the plate was washed as previously. During the washing cycle the substrate solution was prepared depending on the enzyme label used. To each well 200 μ l of

substrate was added. The reaction was stopped in one PBS control well by the addition of the appropriate inhibitor, and this was used to set the photometer absorbance scale to zero. After a time interval of about 15 min the reaction rate in the wells containing the reference positive serum was carefully monitored until it reached an optical density of approximately 0.75. At this point the reactions in all the other wells were stopped.

Each sample was then transferred to the photometer cuvette and the optical density value recorded on the printer. The results were expressed simply as the optical density value recorded.

The diluted serum samples were retained at 4°C so that replicate tests could be carried out.

e) Reagents and buffers for use in the test

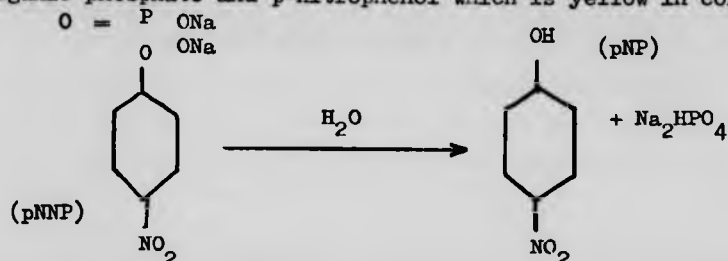
Engvall & Perlmann (1972) in their initial studies on the development of the assay looked at the conditions necessary for antigen binding. In addition to protein concentration, pH was also found to be a critical factor affecting both the percentage uptake and irreversibility of antigen binding. Extremes of pH and the presence of detergent in buffers resulted in loss of antigen from the polystyrene surface. To obtain optimum antigen coating a carbonate/bicarbonate buffer of low molarity and alkaline pH was used.

To prepare dilutions of test sera and conjugate, phosphate buffered saline containing a wetting agent Tween acts as a detergent to reduce non-specific binding of gamma globulin and conjugate during the incubation steps. It is also included in the washing solution. Some workers have included additional protein such as albumin to the incubation buffer to further reduce non-specific binding, but this addition was found to be unnecessary. Bovine serum albumin was, however, added to incubation buffer for the elution of filter paper blood spots to improve antibody stability by increasing protein concentration.

Enzyme substrates

Several factors govern the choice of substrate for use with a particular enzyme antiglobulin conjugate, these are chromogenic properties, chemical safety, ease of preparation and cost.

For alkaline phosphatase, p-nitrophenyl phosphate (pNPP) was found to be a very convenient substrate. The substrate is supplied commercially in tablet form and is readily soluble in diethanolamine buffer. Alkaline phosphatase catalyses the hydrolysis of pNPP to inorganic phosphate and p-nitrophenol which is yellow in colour.



In alkaline solution pNP ionises to p-nitrophenolate anion which has a sharp absorption peak at 400-405 nm. The addition of strong alkali intensifies this colour and also terminates the enzyme-substrate reaction.

The substrates currently available for use with peroxidase conjugates are somewhat less satisfactory. They are usually aromatic amines to which hydrogen peroxide is added as an oxidising agent, with the HRP acting as an oxidation catalyst.

5-amino salicylic acid was the first substrate to be used for peroxidase. It has the disadvantages of being unstable and partially insoluble which can distort the absorbance readings. A more satisfactory substrate was ortho-phenylene diamine which produces an orange coloured end product, with absorption peaks at 464 nm and 492 nm after enzyme substrate inhibition with strong acid. The major drawback of this substrate for routine use is its possible carcinogenic properties.

Details of the preparation of buffer solutions are presented in the appendix.

f) An examination of different batches of commercial microtitre plates as inert supports for antigen absorption

The quality of the polystyrene surface of the microtitre plate used in the test is an important factor governing the quantitative uptake of soluble antigen and its distribution within the well

Table 4. Quality control studies on different batches of microtitre plates

Plate code	No. serum samples tested	Mean OD \pm S.E.	't' paired (p 24d.f.)
Dynatech 129A (control)	25	0.57 \pm 0.09	
Dynatech 129B	25	0.82 \pm 0.11	7.11 (p < 0.001*)
Gibco-Biocult 29A	25	0.69 \pm 0.10	0.72 (p > 0.01)
Gibco-Biocult 29B	25	0.60 \pm 0.08	2.10 (p < 0.05*)
Flow 76-301-05	25	0.61 \pm 0.09	3.60 (p < 0.01*)
Flow 76-201-05	25	0.55 \pm 0.08	1.08 (p > 0.05)

Data were analysed by the paired 't' statistic, each series of OD values being compared with those obtained on the control plate.

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of the plate. Broadly speaking microtitre plates are supplied in three forms, a non sterile plate which is packaged straight after processing, a sterile irradiated plate and a tissue culture treated plate, which is also sterile. The essential difference between the last two types of sterile plate is that the tissue culture plate has been carefully washed after processing to remove traces of plasticiser used in the production process (personal communication, Flow Laboratories). Since the tissue culture plates are expensive most enzyme assay work has been based on the non sterile and sterile irradiated plates. In the early days of enzyme assay work problems were encountered with the quality of microtitre plates and considerable variation was observed between different batches of plates within the same commercial code number. Cheesum & Denmark (1978) showed that the quality of the microtitre plate could greatly affect the quantitative reproducibility of test measurements. Manufacturers have attempted to improve standardisation of microtitre plates for enzyme assay work, and as part of the quality control studies here a survey of some of the plates currently marketed for ELISA was carried out.

Six different types of polystyrene microtitre plate were selected for the study, they consisted of two marketed by Dynatech Laboratories (M 129 A & B), two by Gibco Biocult (M 29 A & B) and two by Flow Laboratories (Linbro 76-201-05, 76-301-05). Each plate was treated in exactly the same way. Soluble egg antigen at its working concentration of 2.5 $\mu\text{g}/\text{ml}$ was coated on to each plate and incubated overnight. A random collection of positive and negative sera were screened at a dilution of 1/300, and their optical density values recorded for each plate when the reference serum on each plate had reached an OD level of 0.75 following the standardised enzyme assay procedure. The paired t statistic was used to test for significant differences between absorbance values obtained on the standard plate with those obtained on the test plates.

As the results in table 4 show there was good quantitative agreement between the different batches of non irradiated plates and the standard M129A plate, but all batches of irradiated plates were significantly different. An examination of individual values revealed most of these differences to occur in the high absorbance range, which tended to be greater on the irradiated plates. The

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Table 5. Stability of antigen precoated plates. Quantitative reproducibility of antibody response on plates stored for 2 weeks and 1 month

Antigen coating	No. serum samples tested	Mean OD \pm S.E.	't' paired (p 24d.f.)
Control plate	25	0.50 \pm 0.06	
2 wk vacuum dried	25	0.46 \pm 0.11	1.49 (p > 0.10)
1 mth vacuum dried	25	0.55 \pm 0.06	2.56 (p > 0.02*)
2 wk 37°C dried	25	0.46 \pm 0.04	1.33 (p > 0.10)
1 mth 37°C dried	25	0.53 \pm 0.06	0.81 (p > 0.10)

Paired comparisons were made with the control, freshly coated plate.

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difference between irradiated and non-irradiated plates may reflect differences in the plates capacity to adsorb antigen. The irradiated plate may have a higher percentage uptake of antigen than the non-irradiated plate and this would influence antibody binding in those samples with high concentrations of specific antibody. Clearly if comparisons of enzyme assay results are to be made between laboratories it is important that the microtitre plate used in the test is carefully standardised.

g) Tests on the stability of antigen precoated plates

One development which would facilitate the use of enzyme assays as field techniques and would shorten their operation time would be the use of precoated antigen plates. Although various workers have kept antigen solution in the plates for several days there have only been a few attempts to prepare dried precoated plates. In this study plates were coated with antigen and then dried by two different methods and stored.

A random selection of positive and negative sera were screened in the antigen precoated stored plates at intervals of two weeks and one month storage and a control freshly coated plate. The precoated plates were prepared in the following way, after overnight incubation the plates were emptied of residual antigen solution and rinsed with washing buffer. They were then dried in one of two ways, either for several hours in an incubator at 37°C or under vacuum for 30 mins. After drying they were sealed with an adhesive cover and stored in plastic bags in a polystyrene box at room temperature.

The paired t statistic was used to test for significant differences in antibody level measured on the control, freshly coated plate and precoated 37°C dried plates and vacuum dried plates stored for periods of two weeks or a month.

As illustrated in table 5, with the exception of vacuum dried plates stored for one month there was no significant difference in absorbance levels between the control plate and precoated 37°C dried plates stored for two weeks and one month and vacuum dried plates stored for one month. Examination of individual absorbance values did however show an increase in non specific background in negative

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sera on the dried plates, which could be important if dried plates were used in a routine survey as false positive reactions could arise through increased non specific background reaction.

h) Tests on the quantitative reliability of using filter paper blood eluates in the assay

The collection of blood samples on absorbent filter paper provides a convenient means of obtaining test material especially in large scale field surveys or when venipuncture may not be acceptable as with small children. The technique has been used very successfully in the indirect fluorescent antibody test for a number of parasitic infections, for schistosomiasis by Anderson et al. (1961). for sero-epidemiological surveys for malaria (Bruce-Chwatt et al., 1973) and for Chagas disease (Voller et al., 1975).

The stability of antibody when absorbed on to paper has also been studied in some detail. Thaver & Draper (1974) investigated the stability of specific malarial antibodies in the IgG and IgM class in relation to type of absorbent paper used and the effects of storage and temperature. Their studies revealed Whatman chromatography paper number 3 to be the most reliable type of absorbent paper, and the greatest stability of antibody titre was achieved at temperatures of below -20°C for periods of up to six months.

Loss of antibody activity was related to humidity and temperature although for practical field purposes carefully dried samples could be preserved for several months at 4°C. IgM antibodies were particularly susceptible to the effects of temperature and humidity and marked drops in titre were observed with increases in both temperature and humidity. This can be an important factor if specific IgM detection is to be used as an indicator of recent primary infection.

In this study the method of blood collection developed in the Ross Institute by Bruce-Chwatt et al. (1973). For screening in the assay a calibrated punch was used to cut out a disc equivalent to 30 µl of blood. This was then placed in a tube containing 0.75 ml of elution buffer to give an approximate serum dilution of 1/50.

sera on the dried plates, which could be important if dried plates were used in a routine survey as false positive reactions could arise through increased non specific background reaction.

h) Tests on the quantitative reliability of using filter paper blood eluates in the assay

The collection of blood samples on absorbent filter paper provides a convenient means of obtaining test material especially in large scale field surveys or when venipuncture may not be acceptable as with small children. The technique has been used very successfully in the indirect fluorescent antibody test for a number of parasitic infections, for schistosomiasis by Anderson et al. (1961), for sero-epidemiological surveys for malaria (Bruce-Chwatt et al. 1973) and for Chagas disease (Voller et al., 1975).

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Table 6. A quantitative comparison of antibody levels from whole blood samples eluted from filter paper and serum samples collected by venipuncture

Samples	No. tested	Mean OD \pm S.E.	't' paired (p 24d.f.)
Blood spots	25	0.57 \pm 0.08	0.95 p > 0.30
Serum	25	0.70 \pm 0.13	

Table 7. A quantitative analysis of the stability of antibodies on absorbent paper following storage at -20°C for 1 year

Blood spots	No. tested	Mean OD \pm S.E.	't' paired (p 24d.f.)
Control	25	0.64 \pm 0.02	3.36 p < 0.002
1 yr storage	25	0.45 \pm 0.01	

25 matched blood spots, collected on filter paper, and serum samples collected in capillary tubes were used in this study. They had been collected in the Sudan from known infected patients, and the method of transport and storage was the same for both sets of samples.

ELISA results for the two groups of samples are presented in table 6. The paired t statistic was used to test for any significant difference between method of blood collection and amount of antibody measured in the test. As the t values show there was no significant difference between the two methods of collection and quantitative antibody levels.

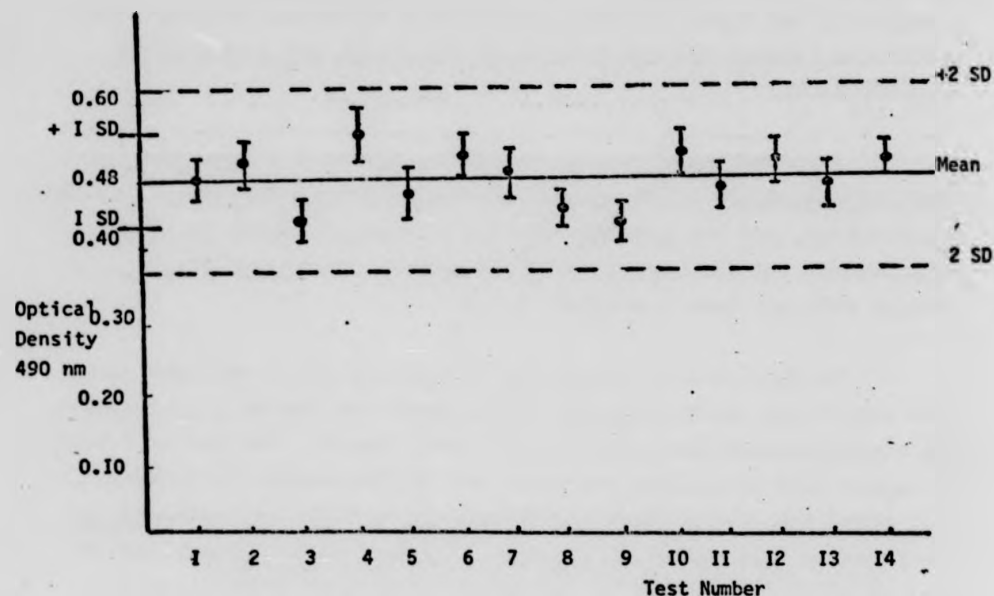
A second study was undertaken to examine the long term stability of stored blood spots. Although Thaver & Draper, (1974) had demonstrated that the IgG antibody titre remained stable in blood spots stored for periods of up to six months, longer periods of storage have not been examined.

In this study a comparison of antibody level was made on 25 duplicate blood spots collected in the Sudan and tested a year apart. The stored samples were kept at -20°C until tested. The paired t test was again used to analyse the data, and as the results in table 7 show there was a significant difference ($p < 0.002$) in antibody level during this period of storage. The most marked change was the loss of antibody activity in originally high titre sera. Loss of antibody activity also affected a number of weak positive sera resulting in their conversion from seropositive to negative.

i) An assessment of the quantitative reproducibility of test measurements and development of quality control procedures

One of the major problems of standardisation in enzyme immunoassays is the need to perform an enzyme substrate reaction, and this is the chief drawback compared to RIA procedures because the conditions under which the reaction is carried out have to be carefully controlled to ensure reproducibility from test to test (Booth, 1977). Several studies have been carried out to establish the limits of 'precision' of enzyme immunoassays, that is the extent of variation in a series of assays performed on the same sample. There are essentially two estimations of precision in an immunoassay and these are usually

Figure 6 . Shewhart Quality Control Chart



Each point on the graph represents a mean based on three replicate readings of the control positive serum. Standard deviations about the mean provide a measure of 'within' day variation of the assay.

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expressed as the coefficient of variation (cv) which is a ratio between the standard deviations of the individual determinants and the mean. A coefficient of variation can be obtained for 'within assay' variation, determined by testing one sample many times in one assay which tends to give an over estimation of precision, and secondly for between assay variation which is based on testing one sample in a number of independent assays usually performed on different days (Schuurs & Van Weeman, 1977).

The need to establish the precision of measurements in an assay lies at the centre of quality control schemes. These are designed with the aim of improving the quality of laboratory results so that uniformity exists within a laboratory and between laboratories. The first aim serves two functions, it enables the laboratory worker to know how well the test results keep within accepted limits and secondly it enables corrections to be made in the assay if variation becomes too wide.

Several quality control procedures exist and can be easily applied to immunological assays. The Shewart quality control procedure was adopted here (Batty, 1977).

An aliquot of the control positive serum, prepared by pooling several positive sera, was screened on ten separate occasions in the assay and the mean and +1, +2 standard deviations calculated. These values were used to construct the Shewart chart which is illustrated in figure 6. In every subsequent assay replicates of the control positive serum were included and the mean and standard deviations calculated. These were plotted on to the chart so that an estimation of test reliability could be established.

As the figure shows, the mean and standard deviation obtained in different tests for the control positive serum fell well within the accepted 95% confidence interval and often within one standard deviation, indicating that the assay procedure developed could be operated to give reproducible results over a period of time. For a further check on the quantitative reproducibility of repeated samples duplicate sets of data from a routine serological screen of several thousand Sudanese blood samples were tested by regression analysis. A positive correlation coefficient of 0.98 was obtained for pairs of ELISA readings, showing the high degree of test reproducibility.

4. Class Specific Antibody Measurements in Indirect ELISA

The levels of serum immunoglobulins represent a balance between synthesis and catabolism controlled by both genetic and environmental factors, the main environmental factor being antigenic challenge. In infectious diseases IgM antibody characterises the primary response to antigenic challenge so that many acute infections are characterised by a rise in IgM. This has been used to advantage for early diagnosis of viral infections such as cytomegalovirus and rubella (Voller, 1975).

A rise in IgM is also characteristic of acute malaria infections, which decline after a few weeks of infection and then IgG becomes the predominant antibody. Raised IgM is not only confined to the acute stages, as it has been known to occur in chronic forms of both malaria and trypanosomiasis, particularly in cases of tropical splenomegaly (Ziegler & Sturver, 1972) and may represent persistence of the primary response in the face of new antigenic challenge exhibited in the form of antigenic variation (Thompson, 1974).

The use of labelled monospecific antisera in indirect antibody assays has been of great value in enabling a distinction to be made between total increases or decreases in class specific antibody response and those directed specifically to parasite antigenic determinants. This distinction is important in parasitic infections since many are capable of inducing a condition of hypergammaglobulinaemia in the host, where only a small component of the total antibody increase within a specific immunoglobulin class is directed towards the parasite (Cohen, 1974). Hypergammaglobulinaemia is a characteristic feature of S. mansoni infection. Elevations of both IgG and IgM are common, but Freeman (1970) estimated that over 95% of the increase in serum IgG was not directed towards the parasite, a finding confirmed by Sher, (1977) in experimental studies of schistosomiasis in mice.

Although the role of specific antibodies in schistosome infections is still unclear, there have been attempts to look at specific immunoglobulin responses. Hult et al., (1975b) and Deelder et al., (1975b) measured specific IgG, M and IgA antibodies to schistosome antigens in patients with active S. mansoni infection. They used two different indirect labelled antibody techniques, the IFAT and DASS.

Using monospecific antisera labelled with an enzyme such measurements can also be made by the indirect labelled antibody technique, but as with other types of assays certain considerations have to be taken into account before the measurements of specific antibody can be made. The results of sandwich assays with Ig class specific labelled antibodies have to be interpreted with some care since the serum may contain specific antibodies of more than one Ig class which can compete with each other for the limited number of antigen sites on the solid phase. The extent of interference is determined by the relative concentrations and avidities of the antibody populations in the various Ig classes. Evidence for the existence of competition in an enzyme linked assay is limited, but Holmgren & Svennerholm (1973) found evidence for antibody competition in work carried out on class specific measurements in Vibrio cholerae.

In this study experiments were conducted to test for competition in the assay in two ways; firstly by absorbing the serum with Staphylococcus aureus protein A, which preferentially binds the Fc receptor of the IgG molecule (Forsgren & Sjoquist, 1966) and has been used satisfactorily for the selective removal of IgG from sera from patients with toxoplasmosis (Chantler et al., 1976) and secondly by selective absorption with anti-IgG bound to preactivated agarose beads (Affi-gel 10) using the principle of antibody purification by gel matrix immunoabsorbents (Guesdon & Avrameas, 1976).

a) Determination of conjugate specificity and working dilutions

For antibody measurements in different immunoglobulin classes to be made reliably in a test, the specificity of the antisera used in the conjugate is important. Gel diffusion analysis of a monospecific (heavy chain specific) enzyme labelled conjugate against the appropriate immunoglobulin class is used routinely to check the specificity of the antisera; a single line of identity between antigen and antibody corresponding to a particular class (IgG, IgM, IgA) indicating monospecificity. As a further check on the specificity of the conjugates used in this study experiments were also carried out in the indirect ELISA, which is a more sensitive system compared to gel diffusion.

Standard IgG at a concentration of 100 ng/ml protein A was coated on to ELISA microtitre plates and left covered at room temperature overnight. After washing, reciprocal dilutions (1/100-1/2000) of peroxidase labelled IgG and IgM conjugates were added to the plate. Following the addition of enzyme substrate the reaction was left to develop for 30 mins before stopping. Only the peroxidase anti-IgG conjugate reacted, yielding high OD levels of above 1.0. No reactivity was observed with the other class specific conjugate, OD levels were generally less than 0.10.

With the exception of anti-IgG conjugates, where the working dilution was established by a single sandwich assay against IgG, the working dilutions of specific IgM was based on tests with positive and negative sera in the standardised assay. A control positive and negative serum were each screened at a dilution of 1/300 against reciprocal dilutions of IgM conjugate (1/100-1/2000). The dilution giving an OD value of about 1.0 for the control positive serum and less than 0.25 for the negative after reaction development for 30 min at room temperature was selected as the working dilution for routine use; for IgM this was 1/500.

b) Tests for selective IgG absorption using *Staphylococcus aureus* protein A

The objective of this study was to examine the effects of absorption of serum with protein A on the specific levels of IgG and IgM antibody measured by ELISA. It was designed to determine whether appropriate absorption was effective in removing IgG without impairing the ease of specific IgM detection, and further if there was any evidence for competition in the assay.

S. aureus protein A was provided by the Dept. of Medical Mycology, L.S.H.&T.M. and prepared following the method described by Anherst et al. (1974). A group of ten serum samples were selected and a 1/50 dilution of each prepared in incubation buffer. To a 0.50 ml aliquot of each serum sample, 0.1 ml of a 10% suspension of protein A was added and the samples mixed at room temperature for 30 min. After thorough mixing they were centrifuged at 2,500 g for 20 min and the supernatant decanted for testing.

Table 8. IgG and IgM levels measured by ELISA before and after
absorption with *S. aureus* protein A.

Serum No.	Absorption	OD 490 nm	
		IgG	IgM
1	-	0.52	0.35
	+	0.11	0.13
2	-	2.00	0.68
	+	0.94	0.46
3	-	2.00	0.89
	+	1.10	0.56
4	-	0.39	0.17
	+	0.25	0.16
5	-	0.31	0.29
	+	0.06	0.26
6	-	0.56	0.36
	+	0.14	0.19
7	-	0.80	0.49
	+	0.14	0.33
8	-	1.17	0.44
	+	0.18	0.42
9	-	1.25	0.30
	+	0.39	0.17
10	-	0.80	0.25
	+	0.35	0.18
		t = 5.65 p < 0.01 t = 4.11 p < 0.05	
		df 9 df 9	

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ELISA tests were carried out on the preabsorbed and absorbed sera at dilutions of 1/300, using peroxidase labelled monospecific antihuman IgG and IgM conjugates (Miles Laboratories). The enzyme substrate reaction was stopped after development for 30 min at room temperature by the addition of 8N H₂SO₄.

Results of specific IgG and IgM levels before and after absorption with protein A are shown in table 8. As the data show protein A effectively depletes the levels of IgG in the serum, reflected by the very significant drop in the OD levels after absorption. There was also, however, a drop in the specific levels of IgM antibody after absorption, but this was less marked than the IgG.

c) Tests for selective IgG absorption using specific anti-IgG coated agarose beads

A second method of removing IgG from serum, using anti-IgG bound to activated agarose beads, was undertaken in an attempt to find a more specific method.

1.0 g of preactivated agarose beads were suspended in 25 ml of 0.1M phosphate buffer pH 7.0 containing monospecific antihuman IgG at a concentration of 5-10 mg/ml, and mixed gently at 4°C overnight. The suspension was then transferred to a Buchner funnel and washed thoroughly with phosphate buffer until the absorbance level at 260 nm reached 0.01. During coupling N-hydroxy succinimide, which absorbs strongly at 260 nm, is replaced by the ligand (antibody) and its removal from the gel solution indicates completed washing. To block remaining active sites in the gel medium, the gel was resuspended in 1M tris, which having a high amino group content effectively inhibits further coupling. This step is important to prevent any antibody in the serum binding to the gel medium during incubation. The gel was left in tris buffer overnight and then washed thoroughly with fresh phosphate buffer and stored at 4°C until needed.

For absorption 1.0 ml of an even suspension of beads was pipetted into each compartment of a flat plate (Repli dish). The beads were allowed to settle and the buffer layer carefully removed by aspiration. A batch of ten serum samples were diluted to 1/300

Table 9. IgG and IgM levels measured by ELISA before and after
absorption with anti-IgG bound agarose beads

Serum No.	Absorption	OD 490 nm	
		IgG	IgM
15	-	0.35	0.27
	+	0.15	0.13
16	-	0.84	0.26
	+	0.27	0.23
17	-	0.74	0.26
	+	0.25	0.15
18	-	0.76	0.18
	+	0.31	0.15
19	-	1.01	0.26
	+	0.73	0.21
20	-	0.84	0.29
	+	0.36	0.21
21	-	0.88	0.26
	+	0.47	0.21
22	-	0.90	0.49
	+	0.40	0.33
23	-	0.31	0.25
	+	0.20	0.23
24	-	0.66	0.36
	+	0.27	0.31
		t = 8.38 p<0.01 df 9	t = 4.65 p<0.05 df 9

and 0.50 ml of each sample added to a separate compartment and mixed into the gel layer. When all samples had been added the plate was placed on a microtitre plate shaker at low speed for 1 hr. Final absorption was achieved by leaving the samples at 4°C overnight.

Prior to screening in the assay the absorbed sera were carefully removed from above the gel layer. ELISA tests were carried out as in the previous study using peroxidase labelled class specific antibody conjugates.

Results of specific IgG and IgM levels before and after absorption are shown in table 9. The anti-IgG coupled to agarose beads provided a very effective immunoabsorbant for the removal of IgG from the serum, as shown by the marked drop in OD levels before and after absorption. There was some drop in IgM levels but the observed OD changes were fairly small.

The results of these experiments have shown both protein A and bound anti-IgG to provide an effective method for reducing the levels of serum IgG. Both methods did, however, cause a drop in specific IgM levels, which was most pronounced with the protein A. Chantler et al. (1976) in their absorption studies observed similar findings with protein A.

The absence of significant increases in specific IgM levels following absorption, while not conclusive in view of the limitations of both methods in 'selectively' removing IgG, would tend to indicate an absence of competitive antibody binding in the assay between different Ig classes. No preabsorption of sera was therefore felt necessary in class specific antibody studies. Non specific IgM measurements can also occur in labelled antibody tests in patients with rheumatoid factor, an IgM auto-antibody. This was not observed to be a problem with the control sera tested and so absorption with IgG coated particles was not felt necessary.

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5 p<0.05

CHAPTER III

EPIDEMIOLOGICAL APPLICATIONS OF ELISA

The work covered in this chapter formed the basis of a detailed study to evaluate the potential of ELISA for sero-epidemiology of schistosomiasis. This was designed to look not only at the performance of the test in relation to important diagnostic criteria but also to assess the relevance of antibody measurements for studying epidemiological problems.

The work is grouped into three broad categories; part one describes studies undertaken to evaluate ELISA as a diagnostic test, part 2 describes the application of the test for prevalence and incidence monitoring following a pilot control programme in a S. mansoni hyperendemic region of the Sudan and part 3 describes the application of ELISA for the detection of S. mansoni infection in the St. Lucia control project, in particular a study of the role of antibodies for monitoring incidence rates in the closing stages of a control scheme when prevalence rates are very low.

1. An evaluation of diagnostic properties
- a) A study of antibody levels in Kenyan school children living in S. mansoni endemic and non-endemic areas.

A survey was carried out in two districts of Kenya in 1969, Kapsabet an area known to be free of schistosomiasis and Machakos which is hyperendemic for S. mansoni. School children from both areas were examined parasitologically for ova and other helminth parasites by the merthiolate iodine formalin concentration technique (M.I.F.C.) and the formol ether sedimentation technique (Goedbloed, 1970), and for immediate hypersensitivity responses by skin tests. Serum samples were also collected by venipuncture and sent to the Ross Institute for testing. They comprised 97 samples from school children living in the Kapsabet area, collected in March and October. None of the children were infected with S. mansoni but most were infected with hookworm, taenia and ascaris. 122 serum samples were also collected from a group of school children living in the S. mansoni endemic area

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Table 10. Skin tests and serology in a survey of school children from Machakos, an area endemic for *S. mansoni*, and Kapsabet, a non-endemic area

Serum samples	No. tested	% positive by				
		Skin tests	CFT	IFAT	ELISA	
			worm	egg		
Machakos all infected with <i>S. mansoni</i>	122	100	97	95	91	96
Kapsabet 3/69	97	65	43	45	-	5
10/69	97	59	-	56	37	10

Skin test - W.H.O. standard technique for immediate hypersensitivity with adult worm antigen

CFT - Complement fixation test

IFAT - Indirect fluorescent antibody test

ELISA - Enzyme linked immunosorbent assay

of Machakos. Parasitological examination revealed all the children to be infected with S. mansoni.

Prior to the start of this project the sera had been tested by CFT and IFAT (McLaren et al... 1978) and stored at -20°C until the present study. Table 10 shows the levels of sensitivity of the tests in detecting known infected individuals and specificity in the uninfected control group. The skin test for immediate hypersensitivity reactions produced a large number of false positive reactions, of the order of 60%, in the control group from Kapsabet. The CFT using both egg and worm antigens and the IFAT also yielded a high proportion of false positive reactions but ELISA had a much higher degree of specificity with a maximum false positive rate of 10% in the control group. Levels of sensitivity were comparable between the tests.

b) Serological responses in groups of patients with a range of heterologous helminth infections

As the last study showed, in evaluating the specificity of the antibody response in an immunological test it is important to select appropriate controls. Some studies investigating the potential of an immunological technique for diagnosis have been weakened by the selection of inappropriate groups for specificity estimations. Umlay et al. (1974) in comparing several serological techniques used sera from Europeans as the control group to estimate false positive reactions in the tests. Since this group would have had no exposure to infections which might cross react (i.e. other helminth infections) in a serological test for schistosomiasis they are likely to give too low an estimation of the false positive rate. Other workers most notably Moriearty et al. (1974) have used groups of people who were parasitologically negative for schistosomiasis but living in a schistosome endemic area as control groups to estimate the specificity of skin tests. Since these individuals were exposed to schistosome infections they represent an unreliable control group and are likely to give an over estimation of the false positive rate, particularly since the parasitological techniques employed for diagnosis are not infallible and infected people can be missed on examination. Ideally control groups of sera should come from areas which are free from schistosomiasis but where other helminth infections, which may cross react in an immunological test, are prevalent.

Table 11. Antibody reactivity in groups of patients infected with helminths other than schistosomiasis

Serum samples	No. tested	No. positive by ELISA/Mean OD \pm S.E.	
New Guinea bancroftian filariasis	75	2	0.39 \pm 0.09
HTD Onchocerciasis	10	3	0.53 \pm 0.07
Strongyloides	6	0	
<u>W. bancrofti</u>			
<u>L. loa</u>	6	3	0.34 \pm 0.07
Ascariasis			
Trichuriasis			
Hookworm	8	1	0.28 \pm 0.00
Total	105	9 (9%)*	

* Percentage seropositive

Communities in the tropics tend also to have higher immunoglobulin levels compared to people living in temperate zones, and this is another reason for selecting control groups from tropical areas for assessing specificity in a test.

The objective of this experiment was to examine several groups of sera from patients with a variety of helminth infections, other than schistosomiasis to obtain further estimations of the specificity of ELISA. Several groups of sera from patients with heterologous helminth infections were examined, they comprised the following:

1. 75 serum samples from cases of bancroftian filariasis in New Guinea
2. 30 serum samples from patients in the Hospital for Tropical Diseases consisting of:
 - 10 cases of Onchocerciasis
 - 6 cases of Strongyloides
 - 6 cases of W. bancrofti and L. loa
 - 8 cases of ascariasis, trichuriasis and hookworm infection.

The results of ELISA on these groups of sera are illustrated in Table 11. Very little cross reactivity occurred amongst the 75 cases of filariasis from New Guinea where there is no evidence of schistosome infection in the human population. There were a greater number of cross reactions from patients from HTD but in most cases the levels of antibody measured were found to be below those found in known cases of S. mansoni infection.

c) A study of the antibody response in a group of patients following an outbreak of cercarial dermatitis in Britain

One of the major problems of false positive reactions in immunological tests are non-specific reactions in people who have been exposed to the cercariae of animal and bird schistosomes (Sadun & Biocca, 1962). While species of animal and bird schistosomes are incapable of becoming established in the human host and giving rise to a patent infection, the cercariae are able to penetrate the skin where they set up a local intense inflammatory reaction. This manifests itself clinically in the form of papular lesions in the skin which occur over most parts of the body exposed to the cercariae.

Table 12. Immediate and delayed hypersensitivity and serological reactions in cases of cercarial dermatitis caused by avian schistosomes

Serum samples	No. tested	% positive by								
		Skin immed cerc. worm		Skin del cerc. worm		CFT	IFAT		ELISA cerc. worm egg	
1. Clinical cases of cerc dermatitis	24	90	20	30	80	65	67	22	0	21
2. Unexposed controls	24	0	0	0	0	0	0	0	0	0

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In 1970 there was an outbreak of cercarial dermatitis in a large number of people who had been swimming in a reservoir in Rickmansworth where water birds were infected with avian schistosomes possibly of the genus Bilharziella (Knight & Worms, 1972). Several immunological tests were used to examine the immune response in a group with skin rashes. In addition to being an interesting immunological study, the sera were also invaluable for determining the specificity of the various immunological tests used, particularly since exposure to avian and animal schistosomes is extremely likely in many tropical areas endemic for human schistosomes, and a large number of false positive reactions to these non-human schistosomes could create a serious limitation to the use of immunological techniques.

Serum samples were collected from 24 individuals in a group with marked skin rashes some 10-30 days after exposure. This group was also tested for an immediate and delayed hypersensitivity reaction following the intradermal injection of S. mansoni cercarial and adult worm antigens. Indirect fluorescent antibody tests were carried out on the serum samples using S. mansoni cercariae as antigen, and also complement fixation tests using infected snail hepatopancreas as antigen, (Knight & Worms, 1972). Antibodies were also measured by ELISA using soluble cercarial egg and worm antigens in the standardised test.

As table 12 shows, there was a very high degree of cross reactivity in the skin tests, both immediate and delayed, the complement fixation test and IFAT using various antigen preparations. ELISA by comparison showed higher levels of specificity with no cross reactions occurring when worm antigen was used in the test.

d) A study of the antibody response in patients infected with heterologous schistosome infections

With the exception of tests such as the circum oval precipitin test (Oliver-Gonzalez et al., 1955c) and the MSA1-radioimmunoassay (Pelley et al., 1977) few serological assays are able to distinguish between species of schistosomes, and this can be a drawback to their use in those areas, chiefly in Africa, where both species coexist and can produce concurrent infections in the host population. The complexity of schistosome antigens accounts in the main for this problem

ELISA
worm egg

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Table 13. Serological reactivity in groups of patients with
S. haematobium and S. intercalatum infections

Serum samples	No. tested	% seropositive/Mean OD \pm S.E.
1. Egyptian adults and children infected with <u>S. haematobium</u>	40	80 0.49 \pm 0.16
2. Gabon adults infected with <u>S. haematobium</u> and <u>S. intercalatum</u>	77	32 0.22 \pm 0.03

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and it is only with the use of highly purified antigens such as MSA1 that this problem can be resolved. In fact cross reactivity between species has been used to advantage by some workers for seroepidemiological studies. S. mansoni antigens were used by Wilkins & Capron (1977) in a serological study of the relationship between egg excretion rates and antibody levels in a Gambian community where only S. haematobium infection occurred.

To investigate the extent of interspecies cross reactivity in ELISA sera from Egyptians infected with S. haematobium and from Gabonese infected with both S. haematobium and S. intercalatum were examined.

Serum samples from 40 Egyptian adults and children (provided by Dr. M. Ismail) living in the Menia area of upper Egypt where only S. haematobium occurs were tested in ELISA and additionally sera from 77 Gabonese adults and children living in an area where only S. haematobium and S. intercalatum occur.

As the results of ELISA tests, expressed in table 13, show there is a range of interspecies reactivity in the assay. The sera from Egyptians infected with S. haematobium shows greatest reactivity with an 80% seropositivity rate. This rate is considerably less in infected Gabonese where only 32% of sera from known infected cases reacted in the assay. The difference in levels of reactivity is probably related to endemicity and intensity of infection, this being much greater in Egypt compared to the Gabon. There is also the possibility that some of the Egyptian patients had been exposed to S. mansoni infection.

2. Sudan project

Molluscicide control programme in the Gezira

The Gezira which in arabic means island is a large plain of some 5 million acres forming a region between the Blue and White Niles. At the turn of the century a major irrigation project was started to channel water from the Blue Nile into a series of canals to feed the land and create new agricultural opportunities to boost the country's economy (Gaitskell, 1959).

The development and expansion of the canal system however provided abundant new habitats for the intermediate snail host of S. mansoni, Biomphalaria glabrata, which in turn resulted in an increase of S. mansoni infection in the human communities living and working in the Gezira area. Greany (1952) in a parasitological survey of the human population living in the region estimated only some 10-15% to be infected, since then prevalence rates have been increasing. Omer et al.. (1976) carried out a parasitological survey in 1973 and found that by the age of 20 years prevalence had reached a peak of 80%. There was also a notable change in the proportion infected with S. mansoni and S. haematobium respectively. Whereas S. haematobium had been the predominant species infecting the human population in the early studies, this had decreased with S. mansoni becoming the predominant species.

Shortly after their survey in 1973 a pilot molluscicide programme was implemented in the Gezira in an attempt to interrupt the transmission cycle of S. mansoni. Molluscicide was applied to all the major and minor canals of the irrigation system in a selected area and its effects on the snail population carefully monitored (Amin & Fenwick, 1977).

Assessment of the effects of the control scheme on infection levels in the human population living in villages in close proximity to the canals was based on the measurement of incidence rate, prevalence rate and intensity of infection. These measurements were made in two broadly divided groups, a) school and village children living in the molluscicide treated areas of the Gezira and b) school and village children living in the non-molluscicide treated areas of the Gezira,

Table 14. A comparison of sensitivity levels in ELISA using soluble worm and egg antigens

Age group	Number tested		% seropositive/mean OD \pm S.E.	
	Worm ag	Egg ag	Worm ag	Egg ag
5-9	358	649	85% 0.58 \pm 0.03	93% 0.70 \pm 0.03
10-14	415	420	93% 0.75 \pm 0.04	99% 0.69 \pm 0.03

which formed the comparison area. During the period 1973-77 two major epidemiological surveys were carried out.

a) A longitudinal parasitological and serological survey from 1973-1977

Two separate surveys were conducted to determine incidence, prevalence rates and intensity levels. The first in school children living in both the molluscicide treated and comparison area, and the second in preschool village children. These groups were chosen as they would provide the most sensitive indicator of changes in transmission. Parasitological diagnosis for ova was carried out in the Sudan by the Kato direct smear test (Teesdale & Amin, 1976). At the start of the survey three separate stool specimens from each child were examined. From each specimen three 25 mg samples were prepared and a quantitative estimation of egg count obtained. An individual was regarded as infected if found positive in any smear but recorded as uninfected if no ova were found in all three smears. Individuals who were diagnosed as negative at the start of the survey were examined at later intervals, and the number of parasitological conversions provided a measure of incidence over a specified time.

Antibody levels were measured by ELISA in school and village children living in both areas. Blood samples absorbed on to filter paper were collected from 1019 school children and 482 village children towards the end of the survey to 1976 and sent to the Ross Institute for testing. Antibodies were first measured to soluble worm and egg antigens and the results are shown in table 14. Both the seropositivity rate and mean antibody level, (OD) were lower with the worm antigen, especially in the younger age group. Soluble egg antigen was therefore selected for further studies because of its greater sensitivity.

A comparison of ova and antibody prevalence rates, based on the cross sectional data for 1976, was carried out and the results are shown in tables 15 and 16 for the Gezira schools and villages respectively. Although only a single blood sample had been collected from each individual in the study groups, by using the results of parasitological diagnosis in the preceding year, it was possible to select a group of probably uninfected individuals and, by measuring the number of serological conversions in this group obtain an estimate of the

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Table 15. Prevalence rates by ova and antibodies for the Gezira villages

Village	Number examined	% ova positive	% seropositive
El Eidad	145	45.5	56.5
Abu Rus *	90	4.4	13.0
Nabti *	82	72.0	76.0
Sharafat	147	47.6	61.0

* Villages in the molluscicide treated area

Table 16. Prevalence rates by ova and antibodies for the Gezira schools

School	Age	Number examined	% ova positive	% seropositive
El Eidad *	5-9	133	77	91
Boys	10-14	64	92	100
El-Eidad *	5-9	34	59	68
Girls	10-14	130	82	94
Bint el Haj *	5-9	45	84	76
	10-14	93	89	92
Wad Sulfab	5-9	55	78	71
	10-14	75	73	93
Kasamir	5-9	93	48	51
	10-14	95	74	82

* Schools in the molluscicide treated area

incidence rate for the period 1975-1976. The village children were selected because prevalence rates were lower and changes in transmission would be more easily detected. Results of this analysis are shown in table 17.

The analysis of cross sectional data based on both parasitological and serological results show very high prevalence rates in both the molluscicide treated and untreated areas of the Gezira, with over 90% of children infected by the age of 10 years in most areas. The high rates of transmission are also reflected in the incidence figures for the Gezira villages. Incidence and prevalence rates tended to be higher by serology probably reflecting the difference in sensitivity of the two diagnostic methods. Teesdale & Amin (1976) estimated the Kato direct smear test to have a sensitivity limit of 40e/g, and hence low intensity infections would tend to be missed and prevalence rates underestimated.

The threshold of sensitivity in a diagnostic test becomes an important criterion for incidence measurements where negative individuals are followed for conversion. Since the absence of ova does not necessarily indicate absence of infection, the use of parasitological tests for diagnosis alone, for the selection of uninfected individuals, could lead to an overestimation of incidence rate by the inclusion of infected people in the 'susceptible' group.

b) A large scale cross sectional survey of school children in 1977

Towards the end of the molluscicide programme in 1977 a much larger cross sectional prevalence survey of the molluscicide treated and comparison area was undertaken. A larger number of schools from both areas were incorporated into the study group in an attempt to reduce the marked variation between schools observed in the earlier study.

The youngest class of school children aged between 7-8 years were examined both parasitologically for ova, as in the first longitudinal study, and serologically by ELISA. Results of prevalence rates by both parasitology and serology are presented in table 18, for the schools covered in the two areas.

As in the previous longitudinal study there is a very marked

Table 17. Incidence rates by ova and antibody conversion in the Gezira villages

Village	No. ova negative 1975	Incidence by ova 1975 - 1976	No. serological conversions 1976	Incidence by ova & serology 1976
El Eidad *	86	17/86 (19%)	8	25/86 (29%)
Abu Rus *	88	3/88 (3%)	8	11/88 (12%)
Nabti *	24	4/24 (16%)	2	6/24 (25%)
Sharafat	102	29/102 (28%)	16	45/102 (44%)
Wad Sulfab	11	2/11 (18%)	1	3/11 (27%)

* Schools in the molluscicide treated area

Table 18. Prevalence rates by ova and antibodies in 1977 cross sectional survey of the Gezira schools

School	No. examined	% ova positive	% seropositive
Bint el Haj*	59	49	55
Azrag	52	36	75
El Sereiha	40	7	23
Maharahara	52	36	61
Reiha	56	17	20
adana	70	19	36
Aidad abu Usha	107	49	70
Hilat Abdad	81	36	63
Falir Omer	82	46	79
Um Merelin	90	55	68
Meilig	92	14	17
Abu Ushar	61	9	31
Hilat Hamad	44	25	55
Total	886	36	52
Sharafat ^c	85	48	68
Wad Sulfab	64	46	67
Kashamir	49	39	55
Hileila	88	24	55
Monslipha Gorshi	38	40	71
Managaza	81	9	43
Nyala	83	17	61
Moussalamia	150	16	23
Tuba el Garoshe	97	36	58
Tagala	41	10	17
Arbiga	210	3	18
Total	986	29	43

* Schools in the molluscicide treated area

^c " " " comparison untreated area

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variation in prevalence rates between different schools within both the treated and comparison area. The overall prevalence figures for the two areas show a lower prevalence rate in the untreated comparison area, 20% and 43% for ova and antibody respectively, compared with 36% and 52% for the molluscicide treated area. These prevalence rates are also considerably lower than those obtained in the previous study where ova and antibody prevalence rates were of the order of 60% and 70% respectively. Again the differences are attributable to school variation, with some schools in the 1977 study having prevalence rates of under 20%.

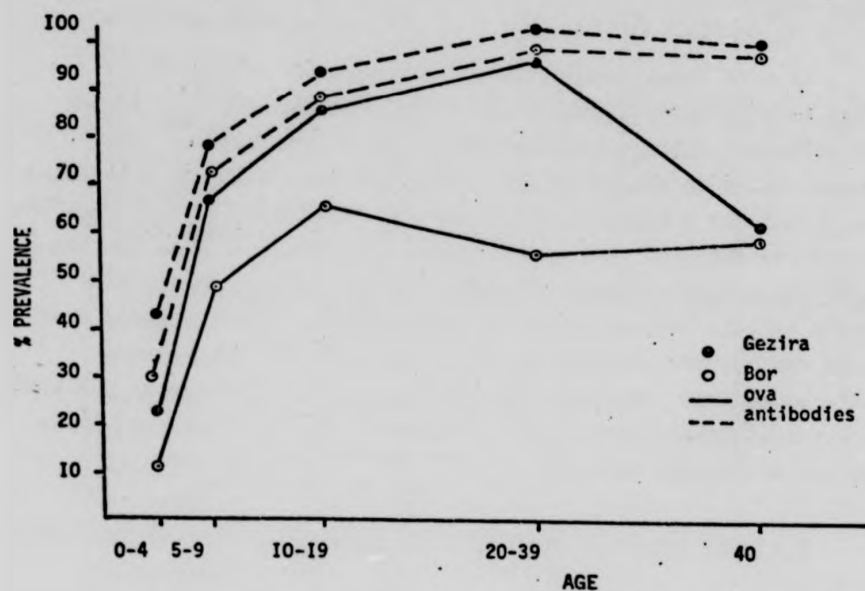
As both these studies show, transmission of infection, measured both parasitologically and serologically, are very varied within different village communities living in the Gezira; this difference being in excess of any difference that could be attributed to the effects of molluscicide in reducing transmission. Although the ELISA results indicate an overall higher prevalence of some 20% compared to the parasitological findings the same trends within the different schools are reflected by both methods. This suggests that serology can provide an accurate reflection of infection status within a community. As mentioned earlier, the differences in positivity rates are most likely because of differences in the detection limits of the two diagnostic methods.

c) A comparison of endemicity of *S. mansoni* in two areas of the Sudan

A study was carried out on samples collected from two areas of the Sudan, the Gezira, which was described in the preceding section, and Bor an area in the Sudd some 700 miles south of the Gezira. The two areas were of interest in that both have endemic *S. mansoni*, but with differences in transmission rates. The objectives of this study were to compare the endemicity of infection, measured by both parasitological and serological methods, and to assess how effectively serology reflects infection levels in the two areas, both in qualitative terms i.e. prevalence of infection, and quantitative terms i.e. intensity of infection.

A total of 1,499 individuals from the Gezira, with ages ranging from under five years to over 40 years were examined for the presence of ova by the Kato direct smear method as described previously, and for total antibodies to soluble egg antigen by ELISA. A second group

Figure 7 . Prevalence of infection in the Gezira and Bor areas of the Sudan



from the Bor area, with a comparable age frequency distribution, were examined similarly.

The age specific prevalence rates for the two areas, expressed as percentage ova and antibody positive, are shown in figure 7. The ova prevalence rates for the Gezira reflect a fairly conventional picture of hyperendemic S. mansoni infection, with a rise in infection levels to a peak of 87% in the 20-39 age group and then a decline to 60% in the 40 and over age group. The serological rate, although at a higher level, parallels the ova rate in children and adolescents but does not show the decline in the older age group. This reflects one of the essential differences between ova and antibody prevalence rates, in that serology generally reflects the cumulative effects of exposure to infection or period prevalence whereas parasitology reflects infection at a given time or point prevalence. With the Bor prevalence data this difference is not apparent because prevalence rates continue to increase with age, both parasitologically and serologically.

The results of this cross sectional survey reflect a difference in the intensity of transmission between the two areas. This is indicated by the overall lower prevalence rates in Bor particularly in the youngest age group. The absence of a decline in prevalence in the older age groups in Bor may also reflect a slower acquisition of resistance to reinfection, although the antibody prevalence rate of over 90% tends to indicate high frequency of exposure.

The quantitative features of S. mansoni infection in the two communities are illustrated in tables 19, 20 and 21. Table 19 shows the distribution of egg counts, broadly divided into three categories, within the two communities. The data are grouped independently of age and show a similar frequency of infection intensity in the two communities, with the majority of the infected population having moderate egg counts. The total antibody levels measured to soluble egg antigen and expressed simply as a mean optical density value, show minimal difference in relation to egg counts. A more detailed examination of the relationship between egg counts and antibody levels, in this case related to age, is presented in tables 20 and 21. There is again a striking absence of an association between the level of antibody measured and the intensity of infection, in any age group, as revealed by the low correlation coefficient values. The one interesting feature of the antibody levels is the shift in the mean peak value from

Table 19. Relationship of antibody levels to intensity of infection,
expressed as egg output, in the Gezira and Bor communities

Egg count	Gezira		Bor	
	Number	Mean OD \pm S.E.	Number	Mean OD \pm S.E.
1-100	335	0.76 \pm 0.08	64	0.74 \pm 0.11
101-1000	403	0.75 \pm 0.06	92	0.76 \pm 0.09
1000	93	0.78 \pm 0.10	24	0.70 \pm 0.07

Egg count on 85% of infected population in Gezira

Egg count on 73% of infected population in Bor

Table 20. Relationship of antibody levels to intensity of infection,
in different age groups, in the Gezira community

Age	Number	Mean egg count Log/egg/g \pm S.E.	Mean OD \pm S.E.	Correlation coeff r
0-4	40	1.38 \pm 0.59	0.98 \pm 0.08	0.15 p > 0.10 ns
5-9	404	1.71 \pm 0.60	0.77 \pm 0.10	0.05 p > 0.10 ns
10-19	341	1.82 \pm 0.63	0.73 \pm 0.03	0.01 p > 0.10 ns
20-39	27	1.74 \pm 0.45	0.78 \pm 0.05	0.31 p > 0.10 ns
40	19	2.00 \pm 0.58	0.80 \pm 0.06	0.36 p > 0.10 ns

Table 21. Relationship of antibody levels to intensity of infection,
in different age groups, in the Bor community

Age	Number	Mean egg count Log/egg/g \pm S.E.	Mean OD \pm S.E.	Correlation coeff r
0-4	5	2.20 \pm 0.84	0.58 \pm 0.11	0.80 p > 0.05 ns
5-9	22	1.73 \pm 0.637	0.50 \pm 0.14	0.14 p > 0.10 ns
10-19	68	1.76 \pm 0.63	0.86 \pm 0.04	0.01 p > 0.10 ns
20-39	66	1.83 \pm 0.71	0.72 \pm 0.08	0.07 p > 0.10 ns
40	19	1.5 \pm 0.61	0.71 \pm 0.03	0.12 p > 0.10 ns

the 0-4 age group in the Gezira survey to the 10-19 group in Bor, which again may reflect differences in transmission.

The results of this study suggest that antibody measurements can provide some useful epidemiological information in schistosomiasis surveys. In children and adolescents antibody levels provide an accurate measure of prevalence of infection, often greater than that obtained by parasitological diagnosis because of test sensitivity differences. The significance of this has already been mentioned with respect to incidence determinations. In adults specific antibodies provide a measure of period prevalence which may be a useful measurement in retrospective studies. The converse is that it does not enable a distinction to be made between active and past infection, and this may be a limitation in some surveys. Perhaps the main weakness to emerge from this study is the absence of any association in this area between total antibody levels and intensity of infection.

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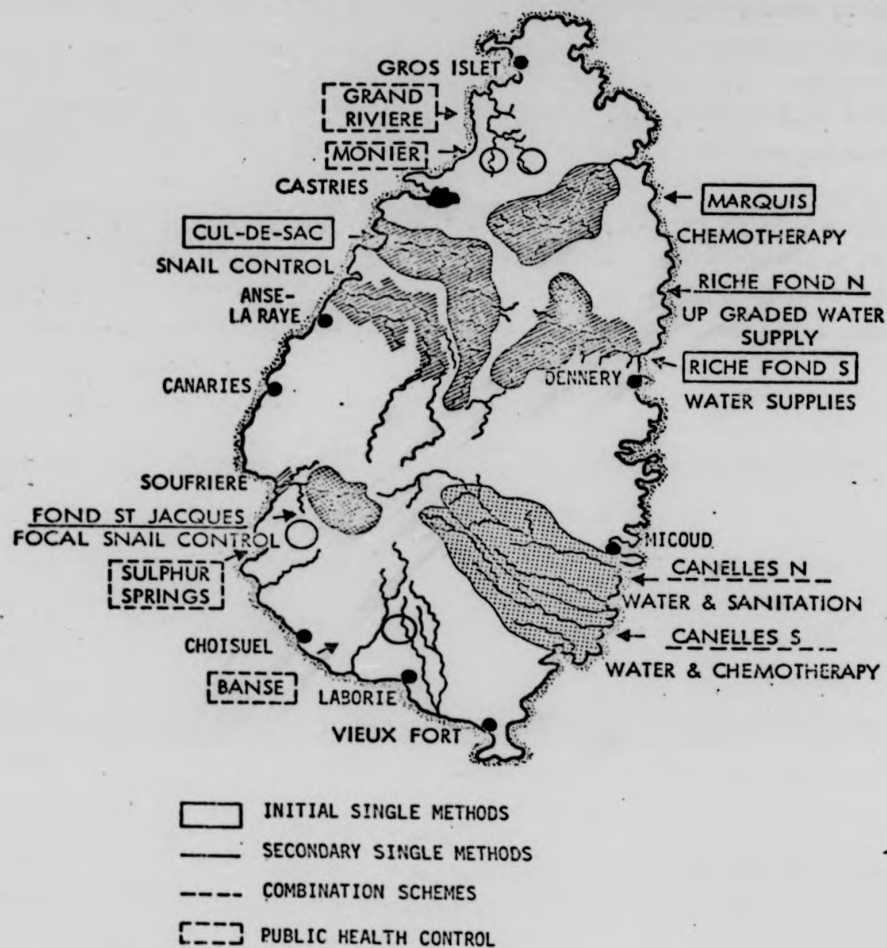
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Figure 8

SCHISTOSOMIASIS CONTROL SCHEME IN St. LUCIA



(Reproduced from Jordan, 1977 Am.J.trop.Med.Hyg. 26,877-886)

3. St. Lucia project

The West Indian island of St. Lucia has been the focal point of a major research project designed to evaluate methods to control the transmission of S. mansoni infection, endemic on the island. The programme was started in 1965 and the objectives were to carry out a comparative assessment of single or combined uses of mollusciciding, chemotherapy and provision of safe water supplies as methods aimed at reducing cercarial densities, contamination and water contact respectively. A map showing the control sites on the island is illustrated in figure 8.

The scheme has been under evaluation since 1970 and details of the results of the difference methods of control reported (Unrau, 1975; Jordan et al., 1975; Cook et al., 1977 and Jordan, 1977). Briefly in all areas under control there was a marked reduction in both incidence and prevalence rates, particularly where chemotherapy had been used. Incidence, prevalence and intensity of infection were used throughout the programme to measure the impact of control on the human population. The measurements were based on results of parasitological examination as the immunological tests tried were found to be lacking in terms of both sensitivity and specificity (Warren et al., 1973).

In areas of high transmission parasitological techniques can be used accurately to measure reductions in transmission following control since, "experience has shown that the higher the prevalence and intensity in the area studied the more accurate are microscopists in their examination of faeces for S. mansoni eggs. However, as prevalence falls the likelihood increases that some of the apparent new infections will actually represent previously acquired light infections which were undiagnosed on examination of a single stool specimen" (Jordan et al., 1975). Over the years the efficacy of the control methods has resulted in very marked reductions in prevalence and incidence rates to the stage where levels are below 5% in several areas of the island (Jordan, 1977), this has created a major problem with respect to diagnosis of infection. In areas where transmission levels are very low detection by stool examination becomes unreliable because of poor sensitivity of the techniques when infection load is low.

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The need for improved diagnostic methods is increasing particularly as the project is now entering the 'consolidation phase'. During the next few years chemotherapy will be progressively removed as a supplementary control method where it has previously been combined with mollusciciding and water supplies, and a careful investigation of changes in prevalence (increase or decrease) will be made (Annual Report, 1978). These studies will in part test the theory behind the hypothetical breakpoint in transmission devised by Macdonald (1959). At the present time it is felt that a cessation of all control measures would probably lead to a resumption in transmission, but that some reduction in costly control, particularly chemotherapy is needed.

The search for new and improved diagnostic methods to measure prevalence and incidence rates is also an important component of the last stages of the present research programme in St. Lucia. In addition to developing better methods of quality control for parasitological techniques (Bartholomew & Goddard, 1978) two serological techniques have been selected for rigorous evaluation, the MSA1-radioimmunoassay developed by Pelley *et al.* (1977) and ELISA.

a) Sensitivity and specificity of ELISA when tested on populations from St. Lucia and St. Vincent

The objective of this experiment was to carry out a qualitative assessment of the assay to detect antibodies in infected St. Lucians of varying age range and infection intensity and a quantitative assessment of the responsiveness of the assay to measure infection intensity. Specificity was tested by examining a population from St. Vincent where schistosomiasis does not occur, but where other helminth infections are prevalent.

A total of 213 male and female patients infected with S. mansoni were examined. They comprised two groups of pretreatment sera, 1) 109 collected during the period '69-'70 and 2) 106 collected between '73-'74. Each serum sample had been drawn by venipuncture prior to treatment and at the same time intensity of infection was determined by taking the arithmetic mean of three stool samples quantitatively analysed by the Bell filtration staining technique (Bell, 1963). All serum samples were stored at -20°C until tested. Intensities of infection varied from 10-200 eggs per gram (e.p.g.) faeces and no patients

Table 22. A comparison of the sensitivity of two antigen preparations used for screening known infected patients from St. Lucia

Serum samples	No. tested	Worm excretory AG	Soluble egg AG
		% seropositive	Mean OD \pm S.E.
1. Infected subjects 1969-1970	109	93 / 0.67 ± 0.06	99 / 0.74 ± 0.05
2. Infected subjects 1973-1974	106	95 / 0.68 ± 0.04	100 / 0.79 ± 0.06

Table 23. Test sensitivity and specificity with soluble egg antigen in *S. mansoni* infected patients and a control group of uninfected St. Vincentians

Serum samples	No. tested	% seropositive / Mean OD \pm S.E.	
		Total Ig	IgG
St. Lucia Group 1 & 2	213	99.5 / 0.79 ± 0.02	98.6 / 0.71 ± 0.05
St. Vincent controls	94	0 / 0.08 ± 0.01	0 / 0.09 ± 0.01

had hepatosplenic schistosomiasis.

For specificity determinations 100 blood samples collected as filter paper blood spots were examined. They consisted of 94 samples collected from St. Vincentian school children aged 6-10 years, and six samples from infected St. Lucians randomly interspersed. The St. Vincentian school children all had a single stool specimen examined for S. mansoni and other intestinal helminth infections. None of them were positive for S. mansoni. 49% had trichuris, 38% had ascaris and 14% had hookworm infections.

All sera were tested in the enzyme assay against soluble egg antigen and some against worm excretory antigen.

As table 22 shows test sensitivity was greatest when soluble egg antigen was used, detecting antibodies in all but one infected individual. The antibody levels were also higher against this antigen as reflected by higher absorbance values (OD). Storage also appeared to have no effect on antibody activity.

There was negligible difference in sensitivity between the measurement of total specific anti-egg antibodies and the specific IgG response, as shown in table 23. Similarly specificity reached the same level of 100% with both antibodies, and the mean OD level of the control St. Vincent sera were well below the diagnostic OD level of 0.25.

The age distribution of antibodies to SEA in the IgG and IgM class are shown in table 24. In the 0-9 age group IgG and IgM antibodies were detected in all infected children, there was then a decline in the numbers with detectable IgM antibody from the 10 year age group onwards. IgG antibody remained detectable in almost all individuals but showed some decline in the > 40 group, accompanied by a drop in antibody level.

Table 25 shows the relationship of antibody levels to intensity of infection expressed as egg output. Analysis by unpaired t test shows a significant difference in antibody levels between the high and low intensity egg excretors and the anti-IgG response, and regression analysis yielded a significant correlation coefficient of $r = 0.183$

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Table 24. Age distribution of antibodies to soluble egg antigen in the
IgG, IgM class in patients infected with *S. mansoni*

Age group	No. tested	% seropositive IgG	Mean OD \pm S.E. IgM
0-9	20	100/0.77 \pm 0.06	100/0.45 \pm 0.03
10-19	81	100/0.72 \pm 0.01	85/0.43 \pm 0.03
20-39	82	99/0.71 \pm 0.02	60/0.40 \pm 0.00
40	30	93/0.64 \pm 0.03	62/0.42 \pm 0.01

Table 25. Relationship of antibody levels to intensity of infection
expressed as egg output and measured against soluble egg antigen

Egg count (o.p.g.)	No. tested	Mean OD \pm S.E.	
		Total Ig	IgG
10-25	71	0.74 \pm 0.03	0.58 \pm 0.03
26-200	114	0.77 \pm 0.02	0.72 \pm 0.02
200	28	0.83 \pm 0.04	0.78 \pm 0.03

$p < 0.05$. No significant difference exists between any of the other groups.

Results from this experiment show that ELISA using a soluble egg antigen can give reliable diagnostic information with sensitivity levels of over 99% and specificity of 100% in St. Lucian St. Vincent populations. The age distribution of specific antibodies in the population reveal most infected people to have detectable levels of IgG antibody but fewer with IgM, except in children; which probably reflects differences in the duration of infection. There is also some association of antibody with infection intensity, the specific IgG response being lower in those individuals with relatively low egg counts compared to those with high egg counts. This is in contrast to the results observed in the Sudanese study.

b) An assessment of quantitative changes in antibody response following effective chemotherapy

The assessment of effective chemotherapy in population studies has almost always been based on results obtained from parasitological examination. A parasitological conversion from ova positive to negative during a period of 3-6 months is generally regarded as a measure of curative treatment (W.H.O., 1966). Immunological methods have been used in a number of studies but results have been very varied and little in the way of practical applications have emerged.

Quantitative changes in the immunological response following treatment would appear to depend on a variety of factors such as, type of drug administered (da Silva, 1976), time interval after treatment (Ambroise-Thomas, 1976) and type and class of antibody measured.

Only two techniques have been able to measure an antibody response which accurately reflects chemotherapeutic cure, the circum oval precipitin test (COP) and the quantitative MSA1-radioimmunoassay. Rifaat *et al.* (1969) showed in their studies with the COPT that antibodies became negative about a month after treatment. The quantitative MSA1-RIA, developed by Hamburger *et al.* (1976) was used on a population in St. Lucia which had undergone treatment as part of the control programme. In their study a large number of those effectively cured were found to be serologically negative 6 months after treatment was

Table 26. Parasitological and serological conversion and decline in anti-SEA antibodies following treatment

Serum samples	Total no. sera	% ova conversions (positive to negative)	% ova negative showing negative in antibody		Soluble egg antigen Mean OD \pm S.E		
			Total Ig	IgG	Total Ig	IgG	IgM
Pretreatment	37				0.87 \pm 0.03	0.75 \pm 0.08	0.38 \pm 0.01
Post treatment							
6 months	31	25/31 = 80%	17/25 = 68%	17/25 = 68%	0.67 \pm 0.06	0.50 \pm 0.07	0.37 \pm 0.01
1 year	21	15/21 = 71%	12/15 = 80%	12/15 = 80%	0.71 \pm 0.10	0.53 \pm 0.11	0.37 \pm 0.07

started, and all were confirmed as seronegative after one year (Annual Report, 1977). Their study was of particular interest because the individuals studied were comparable to those examined in this experiment.

A group of 37 patients with pre- and post-treatment sera were tested in ELISA. All had a quantitative stool examination done prior to treatment and a proportion were subsequently re-examined parasitologically at intervals of 6 months and a year following initial treatment. At the same time as the stool examination a serum sample was collected by venipuncture. Total, IgG and IgM antibodies were measured in the assay. Table 26 shows the proportion of parasitologically cured patients who exhibited a decline in antibody level. This ranged from nearly 70% at 6 months to 80% a year after treatment.

The table also shows the antibody levels in the group of patients before treatment and in a proportion of them at 6 months and 1 year after treatment. The IgM levels were low overall and showed no change after treatment. The total and IgG antibody response showed the most marked change and a more detailed analysis was undertaken.

The quantitative drop in antibody level was highly significant as results of the paired t statistic show, in table 27. However, the proportion of individuals who converted from seropositive to negative was very small, less than 10% at 6 months and about 15% at a year.

c) A study of the antibody levels in hepatosplenic schistosomiasis

One of the main pathological lesions in S. mansoni infection is fibrosis of the liver. This is attributable to an intense inflammatory reaction around ova which become trapped in the liver tissue. These destructive lesions are essentially cell mediated immunologic responses (type IV) to antigen secreted by viable ova. The result is the formation of a granuloma which becomes progressively fibrosed giving rise to hepatosplenomegaly (Warren, 1976).

Epidemiological studies in man have revealed an association between the intensity of infection measured by egg output and prevalence of hepatosplenic disease (Lehman et al., 1976), and this has been substantiated in post mortem studies showing severe disease to be

Table 27. Analysis of quantitative change in antibody levels in paired pre- and post-treatment samples

Serum samples	No. paired samples	Mean OD \pm S.E.	
		Total Ig	IgG
Pre-treatment	25	0.89 \pm 0.09	0.85 \pm 0.15
6 months post treatment		0.58 \pm 0.10	0.45 \pm 0.10
Paired t test		t = 10.21 p < 0.001 16 df	t = 7.78 p < 0.001
Pre-treatment		0.92 \pm 0.11	0.93 \pm 0.38
1 year post treatment	15	0.60 \pm 0.10	0.50 \pm 0.27
Paired t test		t = 9.10 p < 0.001 11 df	t = 4.93 p < 0.01

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associated with heavier worm burdens (Cheever et al... 1968 & 1977). Morbidity studies in St. Lucia have also shown a relationship between hepatosplenic disease and infection intensity (Cook et al... 1974), however it has also been observed that not all infected patients with heavy infections develop hepatosplenic disease and conversely some patients with low egg excretion levels develop clinical disease (Goodgame et al... 1978).

Studies in experimentally infected animals have shown that during the chronic phase of the infection hypersensitivity reactions around the ova are modulated, presumably by immunological factors (Boros et al... 1975). It follows from this that the development of hepatosplenic disease in some patients may represent a failure on a part of the immune response to modulate the hypersensitivity reaction. Of the various mechanisms put forward to explain modulation of the hypersensitivity response the role of antibodies as blocking agents has been suggested (Goodgame et al... 1978).

The purpose of this study was to test in part this hypothesis, to see whether there was any difference in humoral response between patients with or without hepatosplenic disease. In addition to studies with ELISA quantitative antibody determinations were also assayed by the MSA1-radioimmunoassay and also by their ability to suppress antigen lymphocyte blastogenesis. Details of these studies are presented by Goodgame et al... 1978.

Sera from 24 patients with clinically diagnosed hepatosplenic schistosomiasis were collected by venipuncture, each patient had three separate stool samples quantitatively examined by the Bell filtration technique for S. mansoni ova. The group were carefully matched for age, sex and faecal egg excretion with 24 controls living in the same endemic area but without hepatosplenic disease.

Total antibodies to soluble egg and worm antigen were measured by ELISA, and IgG and IgM antibodies to soluble egg antigen using the standardised assay procedure.

Table 28 shows the results obtained with ELISA tests on the two groups of sera examined. The paired t test statistic was used to test for any significant difference in antibody response between

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Table 28. Antibodies to soluble egg and worm antigen in patients with hepatosplenic disease and matched controls

Groups	Number	Mean age (yrs)	Mean egg count egg/g	Total anti-worm	Mean OD \pm S.E. Total anti-egg	IgM anti-egg
Hepatosplenic	24	18.1	231	* 0.81 ± 0.07	0.79 ± 0.09	0.34 ± 0.03
Intestinal (controls)	24	20.2	253	* 0.77 ± 0.06	0.82 ± 0.05	0.35 ± 0.02

* No difference between mean values in any group by paired t test.

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patients with hepatosplenic disease and those with just intestinal schistosomiasis. No statistical difference was observed between the antibody levels in the two groups in any of the antibody classes.

Hypergammaglobulinaemia is a characteristic feature of S. mansoni infections and has been demonstrated in several community studies. Kellermeyer et al. (1973) found a significant difference in the concentration of serum immunoglobulins between St. Lucians infected with S. mansoni and uninfected matched St. Vincent controls, the IgG component in particular was elevated. There have also been various reports on the nature of schistosome induced hypergammaglobulinaemia, some workers have supported the view that it is due to elevations in the specific anti-schistosomal antibody response (Hillyer, 1969; Camus et al., 1977) whereas others support the hypothesis that it is mainly non specific globulin production (Freeman et al., 1970; Sher et al., 1977). Clearly for specific antibody determinations to be made the assay procedure adopted must have an acceptable level of specificity. In their recent study of "specific" antibody levels in Brazilian patients with hepatosplenic schistosomiasis and controls (Camus et al., 1977), the diagnostic assays used were reported to have poor levels of specificity, and thus their study incurred the risk of simply detecting raised levels of immunoglobulin not specifically produced to schistosome antigens.

The use of ELISA in this experiment was felt justified since the assay had acceptable levels of sensitivity and specificity, as illustrated in the earlier experiments. It could therefore be used to indicate whether hypo or hyper-responsiveness was associated with hepatosplenic disease.

The absence of a significant difference in response between patients with hepatosplenic disease and carefully matched controls suggests rejection of the null hypothesis, of an altered immune response with respect to humoral antibody. These results are not, however, conclusive, and it is possible that antibodies may play a role in the modulation of hypersensitivity reactions in liver granulomas. Since the antigen extracts used in this study are a mixture of many different antigenic components, the broad antibody responses measured may in fact have masked an antigen antibody response which has a functional role in modulation. This role might be the suppression of T-cell reactivity to secretory antigens, or the neutralisation of these

antigens through the formation of antigen-antibody complexes.

d) A serological analysis by radioimmunoassay and ELISA of a longitudinal parasitological survey

Several detailed experiments were carried out during a month's visit to St. Lucia as part of the joint project to set up the enzyme immunoassay in the serology unit at the Research & Control Department. The purpose of the exercise was to start a long-term evaluation of ELISA, run in parallel with the MSA1-radioimmunoassay, as part of a search for a sensitive and specific serological test to supplement or replace stool examination for future diagnosis of infection in the control programme.

Equipment for the test was purchased in London and transported to St. Lucia. Basically it consisted of a Vitatron photometer with printer and electric pump, microtitre plates, micropipettes, chemical and immunological reagents. The standardised test procedure was used without modification, and, after a short training period, performed by St. Lucian technicians.

The objective of the first experiment was to compare levels of sensitivity of ELISA with the qualitative MSA1-radioimmunoassay using serum in the form of filter paper blood spots, from a population followed longitudinally by parasitological examination.

326 blood samples from St. Lucian patients who had been examined parasitologically during the period 1968-77 by the Bell filtration technique were tested by both RIA and ELISA. The serum was collected in the form of drops of blood on absorbent paper following the method already described. For the purpose of this study individuals were divided into four groups on the basis of parasitological diagnosis.

1. Individuals repeatedly positive by stool examination.
2. Individuals positive once by stool examination.
3. Individuals consistently negative by stool examination.
4. Individuals with recently acquired infections, within a period of a year.

Antibodies to the MSA1 component of soluble egg antigen were measured in the qualitative MSA1-radioimmunoassay developed by Pelley

Table 29. A comparison of sensitivity between ELISA and MSA1-RIA in four groups of St. Lucian patients

Group	No. tested	Mean no. stool exams	Mean egg count eggs/g	% positive by	
				ELISA	MSA1-RIA
1	157	6.4	24.2	93	97
2	180	6.4	15.6	82	82
3	53	6.7	0	* 40	* 42
4	36	7.0	N-D.	** 97 (100)	** 97 (100)

* Parasitological re-examination of seropositive reactors revealed 48% to be infected.

** The one seronegative in both tests was found to be uninfected on four subsequent stool examinations.

- 1 - Individuals repeatedly positive by stool examination
- 2 - Individuals positive once by stool examination
- 3 - Individuals consistently negative by stool examination
- 4 - Individuals with recently acquired infections, within a period of a year.

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et al., (1977) and to soluble egg antigen in ELISA. The MSA1-RIA had been set up in St. Lucia prior to the start of the ELISA project and was operated by locally trained staff.

Comparative results of ELISA with the MSA1-RIA are presented in table 29. Comparable levels of sensitivity were found between the two tests although ELISA was slightly less sensitive than the RIA. Although the total percentage seropositive in group 2 was identical in both tests, 17% of samples did not match in the two assays, approximately equal numbers were positive in one test and negative in the other. The discrepancy between individuals positive in RIA and negative in ELISA could be explained on the basis of sensitivity differences between the two tests. The ELISA having a lower sensitivity would be expected to produce more false negatives. In both tests the diagnostic cut-off is an arbitrarily defined value, and this may be the cause of other discrepancies. Borderline positive values were common in many of the samples in which equivocal results were obtained.

These results contrasted with the excellent matching between the two tests (over 90%) found with seropositive reactions in group 3, only two of the total of 23 seropositives did not match in the two assays. Group 3 comprised two distinct age groups, 3-18 and 40-73. In both assays the seronegatives showed a bimodal distribution, but the seropositives were clumped in the 3-18 group. Further parasitological re-examination of the seropositives revealed 48% of them to be infected. It is most likely that all the seropositives were infected individuals who had been missed on a single stool examination.

ELISA also matched well with the RIA in tests on group 4 individuals with recent infections. On the basis of stool examination these were either new infections acquired during the period 1976-77 or cases of reinfection. Both assays detected antibodies in all but one individual who on subsequent parasitological re-examination was found to be ova negative on four separate occasions. These results suggest that both assays can reliably detect recent infections, an important factor in relation to future incidence measurements.

e) A cross sectional survey by stool and serology in an untreated population

An analysis of ova and antibody prevalence rates

Over 300 individuals from the small village of La Caye

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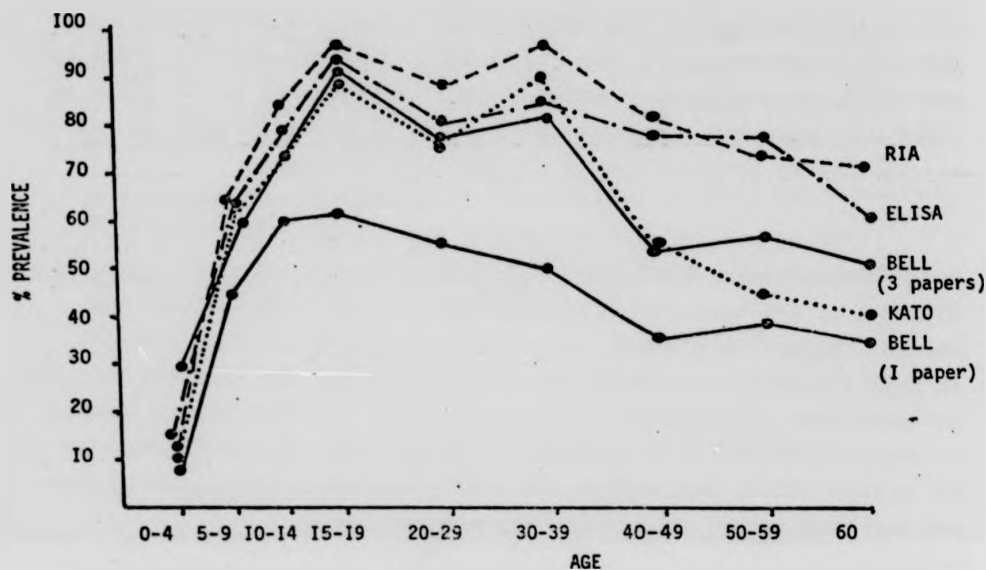
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Figure 9 . Prevalence of infection in a comparative survey of an untreated population by stool examination and serology



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(population 516) in Richefond Valley were examined for the presence of S. mansoni ova by the Bell filtration technique and by the Kato direct smear test and for antibodies by the MSA1-radioimmunoassay and ELISA. The village was selected because it had not been included in the control programme and therefore provided an ideal community in which to compare prevalence estimations by parasitological and serological methods.

The age specific prevalence curves measured by the four tests are shown in figure 9. The figure also shows the difference in prevalence estimations obtained on the examination of one and three stained papers in the Bell technique. The increased sample size results in an overall increase in prevalence rate of some 20%. The prevalence curves for all four tests match closely in children and adolescents with seropositivity rates being slightly higher compared to the ova positive rates. This difference is most likely a reflection of the greater sensitivity of the serological tests. In the 0-4 yrs age group, however, the highest prevalence rate was recorded with the Bell technique when three filter papers were examined. The Bell is unlikely to be more sensitive at detecting infection in this group compared to the other three tests and hence the discrepancy in results is probably due to misdiagnosis of infection through contamination of the apparatus used in the test.

In older individuals there is a greater divergence in the ova and antibody prevalence rates which is generally attributed to the difference between point and period prevalence measured by parasitological and serological methods respectively. Rarely can serological methods discriminate between the antibodies produced in an active infection and those persisting from a previously acquired infection. The antibody prevalence rates do however show some decline in the older age groups which may reflect a decay in the absence of a persisting infection or re-exposure. The possible significance of these findings is discussed later.

A comparative analysis of sensitivity and specificity in the four diagnostic tests

The objective of this study was to carry out a detailed comparison of sensitivity and specificity in the four diagnostic tests

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which consisted of two parasitological techniques and two serological tests.

Although comparative studies have been done in the past on parasitological and serological tests they have generally involved a one-sided assessment of serological tests against the 'reference' parasitological test (Buck et al., 1964; Warren et al., 1973). While this approach has been of value in assessing the reliability of different serological techniques it is based on the assumption that the chosen parasitological 'reference' test yields reliable diagnostic information. There have been relatively few detailed comparative studies on the combined use of serological and parasitological techniques with an evaluation of their qualitative and quantitative reliability.

The purpose of this experiment was to undertake such a study and to use as a diagnostic reference a combination of tests rather than the selection of any one test as a reference.

A total of 304 individuals were examined by all four tests. For parasitological diagnosis a single stool specimen was examined in both the Bell technique and the Kato smear test. The Bell filtration technique which has been the routine method for diagnosis of infection in St. Lucia was carried out by locally trained staff using the original method described by Bell (1963). The Kato direct smear test by comparison was carried out by a highly trained parasitologist using the method described by Kato & Miura (1954). Both serological tests were carried out by locally trained staff under supervision, using the standardised assay procedures already described. In both tests serum in the form of filter paper blood eluates was used.

To compare the diagnostic reliability of the four tests an approach devised by Goddard (personal communication, 1978) based on a modification of Cochrane's Q analysis (Cochrane, 1950) was used. The methodology is based on the conventional χ^2 significance test, but is a more appropriate method to use when data from a number of 2X2 contingency tables is pooled. In this study the statistic was used to

Table 30. Categorisation of data in the four diagnostic tests, Bell, Kato, RIA and ELISA compared to the diagnostic reference

♂ Age		♀ Age		Total	Test results					Agreement with reference			
<10	>10	<10	>10		Bell	Kato	RIA	ELISA	Ref*	Bell	Kato	RIA	ELISA
14	53	14	70	151	+	+	+	+	+	1	1	1	1
0	3	1	5	9	+	+	+	-	+	1	1	1	0
0	0	0	0	0	+	+	-	+	+	1	1	0	1
1	0	2	0	3	+	+	-	-	-	0	0	1	1
1	5	1	8	15	+	-	+	+	+	1	0	1	1
1	1	0	0	2	+	-	+	-	+	1	0	1	0
1	0	0	2	3	+	-	-	+	+	1	0	0	1
3	3	5	5	16	+	-	-	-	-	0	1	1	1
0	11	6	9	26	-	+	+	+	+	0	1	1	1
0	0	1	1	2	-	+	+	-	+	0	1	1	0
1	1	0	0	2	-	+	-	+	+	0	1	0	1
1	0	0	0	1	-	+	-	-	-	1	0	1	1
3	5	1	8	17	-	-	+	+	-	1	1	0	0
1	1	0	0	2	-	-	+	-	-	1	1	0	1
1	2	3	0	6	-	-	-	+	-	1	1	1	0
14	9	13	13	49	-	-	-	-	-	1	1	1	1
42	94	47	121	304									

* Diagnostic reference = positive if one or more of both a parasitological and an immunological test positive

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compare the performance of each diagnostic test in relation to a selected diagnostic reference. This was defined as "an individual was regarded as infected if simultaneously positive in one or more of both a parasitological and immunological test".

The data were divided into the following categories: male and female under and over ten years. A frequency distribution of the results obtained in each of the four tests was then plotted for each category as illustrated in table 30. Where a test agreed with the diagnostic reference it was given the notation (1) and where it disagreed the notation (0). Cochrane's Q analysis was then used to test the null hypothesis that there was no difference in the diagnostic capacity of each test, by measuring the agreement between tests in relation to the diagnostic reference. The results are shown in table 31. In common with the χ^2 statistic, a high Q value indicates significance. From the table the Q value for the overall comparison indicates a significant difference between the diagnostic performance of the four tests suggesting rejection of the null hypothesis. This difference appears mainly due to diagnostic errors in females under 10 years.

To examine in more detail the difference in the tests the following null hypotheses were constructed, and again tested by the Q analysis.

1. No difference exists between the diagnostic capacity of parasitological and immunological tests.
2. No difference exists between the diagnostic capacity of the Bell technique and Kato, RIA and ELISA.
3. No difference exists between the capacity of ELISA and Kato and RIA.

Bell and ELISA were selected for further comparison because they had the lowest number of agreements with the diagnostic reference.

The results of the analysis of these three comparisons are shown in table 32. A comparison of paired immunological and paired parasitological tests reveals no significant difference in their diagnostic capacity and serves to validate the selection of diagnostic criteria. Comparison of the Bell technique with the other three tests shows a very significant difference between its diagnostic capacity and

* Diagnostic reference = positive if one or more of both a parasitological and an immunological test positive

42 94 47 121 304

Table 31. Cochran's Q analysis of agreement between Bell, Kato, RIA and ELISA in relation to the diagnostic reference

Group	No. in group	No. of agreements of each test with the reference				Q analysis (Compared χ^2 on 3df)
		A Bell	B Kato	C RIA	D ELISA	
↖10	42	37	37	36	37	0.18 ns
>10	94	79	88	87	83	5.91 ns
↖10	47	33	44	46	41	18.38 p < 0.01
♀>10	121	106	111	111	107	1.99 ns
Total	304	255	280	280	268	15.02 p < 0.01

Table 32. Cochran's Q analysis of agreement between grouped tests

Tests	No. of paired agreements with the reference	Q analysis
A & C	535	1.49 (compared χ^2 1df) ns
B & D	548	
A	255	11.64 " p < 0.01
B & C & D	828	
D	268	4.65 " p < 0.05
B & C	560	

A = Bell
B = Kato
C = RIA
D = ELISA

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that of Kato, RIA and ELISA. From table 31 it is clear that the significant difference in tests is mainly attributable to the poor performance of the Bell technique. It shows the lowest number of agreements with the diagnostic reference in all four categories, but the disparity is particularly wide in the females under 10 years with an agreement of only 33/47 compared with 46/47 for RIA. There is no clear explanation for the very low agreements in this particular category, especially since stool examination is carried out on a random basis. ELISA also emerged as a less reliable diagnostic test compared to RIA and Kato although the difference in diagnostic capacity was less significant.

In order to compare the levels of sensitivity and specificity in the four tests, the method described by Armitage (1971) based on the method of Youden (1950), and illustrated in table 33 was adopted. From the table categories 'b' and 'c' represent the false negative and false positive levels respectively. By combining the sum of these errors on the assumption that they are of approximately equal importance an expression called the 'J' index is obtained. Using this classification system table 34 shows the levels of sensitivity and specificity of the four diagnostic tests compared with the diagnostic reference defined above. The percentage misclassification per 100 positives provides a measure of the false negative rate, that is the number of true infections which are missed by the test, and the misclassification per 100 negatives the false positive rate, in other words the proportion of uninfected people who are diagnosed as infected. The 'J' index is a combination of these factors and provides a relative measure of the reliability of a diagnostic procedure.

The results of the analysis show there to be a considerable variation in diagnostic efficiency between the four tests reflected by marked variation in both sensitivity and specificity as illustrated in table 34. In terms of sensitivity the two serological tests emerged as the most reliable with the MSA1-RIA detecting antibodies in 97% and ELISA in 93% of infected cases based on the diagnostic reference. Specificity levels showed considerable variation with the Kato direct smear test showing greatest specificity with a false positive rate of 5%. Both RIA and ELISA had high false positive rates of over 20%, but a degree of care has to be taken in interpreting these figures. As earlier studies have shown both the RIA and ELISA when tested with

Table 33. Hypothetical fourfold classification of diagnostic and screening test results

Diagnosis*	Screening test		
	+ve	-ve	Total
Infected	a	b	a + b
Uninfected	c	d	c + d
Total	a + b + c + d = n		
Prevalence (Diagnostic)	$= \frac{a + b}{n}$		
Prevalence (Screening)	$= \frac{a + c}{n}$		
Sensitivity	$= \frac{a}{a + b}$		
Specificity	$= \frac{d}{d + c}$		
'J' Index	$= \frac{ad - bc}{(a+b)(c+d)}$		

* Diagnostic reference

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sera from control populations not exposed to schistosomiasis, had very high levels of specificity (Pelley *et al.*, 1977), indicating that false positive reactions in this study cannot be attributed to cross reactive antibody from unrelated helminth infections which have generally been the cause of poor specificity in other serological tests (Warren *et al.*, 1973). The two most likely explanations for the high proportion of serological false positives are firstly, true infected individuals missed on parasitological diagnosis because of a lower threshold of sensitivity compared to the serological tests and secondly serological reactivity in older individuals from a previous infection. Only by very careful parasitological reexamination of the 17 seropositive /ova negative individuals can the true status of infection be ascertained. However, there is evidence from other studies which supports both these hypotheses. In (3d), in the group of individuals who had been diagnosed as consistently negative by parasitological methods 48% were found to be seropositive in both RIA and ELISA and many of these were subsequently found to be infected when more thorough parasitological examinations were carried out. This clearly illustrates the differences in sensitivity between the two methods and provides one possible explanation for the lower specificity of the serological tests. As the age specific cross sectional analysis in figure 9 shows the greatest divergence of ova and antibody prevalence rates occurs in the older age groups, reflecting in part the difference between numbers with active infections and those with antibody from a previous infection.

Although the Kato direct smear test emerged as a very reliable diagnostic method it was unfortunate that the test was not operated under local conditions to make it comparable with the other tests. It is impossible from this study to know whether it would have emerged with such a high degree of reliability if performed by locally trained staff under the same conditions as the other tests.

The Bell technique which has been used as the standard routine diagnostic method emerged as the least reliable test and its performance was significantly lower than the other three tests. Although a proportion of false negatives were anticipated because of its lower threshold of detection the large number of false positive observations was very surprising. Unlike the serological false positive rates which might have been distorted by factors already mentioned it is impossible to regard these parasitological false positives as anything other than

Table 34. Sensitivity, specificity and 'J' index of reliability in the four diagnostic screening tests compared to the diagnostic reference

Screening test	Sensitivity	Specificity	Misclass per 100 +ves	Misclass per 100 -ves	Total	'J' Index
Bell filtration technique	85%	79%	15%	21%	36%	64%
Kato direct smear test	90%	95%	10%	5%	15%	85%
MSA1-RIA	97%	79%	3%	21%	24%	76%
ELISA	93%	75%	7%	25%	32%	68%

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misclassifications of uninfected people. There are no easy explanations for the discrepancies in the test. Contamination of the apparatus used in the technique seems the most likely source of error and also the identification of stained material other than ova on the filter paper.

Both methods used to analyse these results have been based on the use of an arbitrarily defined diagnostic reference, which combines the results of both parasitological and immunological tests. The rationale behind the approach was a decision to treat each test equally, without weighting in favour of any one type of test. This constitutes a quite different approach from earlier comparative studies where only a single parasitological test has been used as a reference. For the diagnostic reference to be valid, however, there must be no significant difference in the diagnostic performance of paired parasitological and paired immunological tests. As the results of Cochran's Q analysis show, there was no significant difference between the performance of the two types of test and hence the criteria chosen could be used as a valid reference.

Although it has certain weaknesses the approach is an attempt to remove the inherent 'bias' in the use of a single diagnostic method for comparative assessment.

A qualitative comparison of other important diagnostic criteria

The quantitative comparative study has provided useful information with regard to the two most important criteria of test sensitivity and specificity. However, as illustrated in figure 1, there are other criteria which have to be taken into account when selecting a diagnostic test for practical routine use and most of these have to be evaluated in qualitative terms. An attempt was made, although at a rather superficial level, to make some comparison between different tests in terms of the type of sample needed in the test, method of collection, complexity of test operation and cost effectiveness.

Both parasitological tests mentioned require the collection of stool samples which necessitates two visits to the villages by the field team, one to deliver the stool cups and the second to collect the following day. Stool collection can become socially unacceptable if repeat visits have to be made to the village and this can lead to the practice of exchanging stool samples which leads to diagnostic errors.

Both serological tests by comparison can use very small quantities of blood (30 ul) collected on to absorbent paper. This involves only one visit to the village by the field team but members have to be trained in the technique of collecting the blood samples.

All the diagnostic methods require trained technical staff to operate the tests, but a higher level of training is probably needed for the serological tests. Both serological tests were, however, mastered within a relatively short period of time, approximately two weeks, by technical staff trained locally.

Detailed information of the cost of the different tests has not been calculated but all require a fairly substantial capital outlay for the purchase of items such as microscopes for the parasitological tests, a gamma counter for RIA and a spectrophotometer for ELISA. As regards recurrent costs serological methods require fewer technical staff to perform the same number of tests as parasitological tests and they can also be performed in a shorter period of time. Serological tests do, however, require more in the way of disposable laboratory equipment and the use of purified antigens in a test constitutes a very important recurrent component influencing cost effectiveness of the test. The need to use purified antigens in a test is amply illustrated by the improved diagnostic reliability of the RIA using the purified egg antigen MSA1.

f) Quality control studies between laboratories

An important aspect of establishing a technique in a new laboratory is to incorporate quality control so that a measure of test reliability can be obtained. For diagnostic assays this needs to be assessed both qualitatively i.e. in terms of copositivity and negativity and quantitatively in the levels of antibody measured in the test.

In standardising the test procedure certain quality control procedures were adopted enabling confidence intervals to be set for test parameters, in particular for determining the accepted limits of error of quantitative readings on repeated samples. To assess the qualitative and quantitative reproducibility of test measurements between the two laboratories the following procedure was adopted. Sets of duplicate blood spots were prepared in each laboratory and one

Table 35. Reproducibility of test measurements in ELISA performed in two laboratories

Sample no.	Mean OD London (ref)	St. Lucia (test)
Positive series		
1	0.70	0.78
2	0.61	0.76
3	0.67	0.65
4	1.13	1.10
5	1.30	1.23
6	0.51	0.54
7	0.96	0.86
8	0.57	0.84
9	0.58	0.80
10	0.26	0.46
11	0.37	0.57
12	1.37	1.43
13	0.54	0.86
14	1.17	1.24
15	0.67	0.95
Mean OD \pm S.E.	0.76 \pm 0.10	0.87 \pm 0.09
t paired	t = 3.19 p < 0.05 for 14 df	
Negative series		
1	0.09	0.16
2	0.03	0.08
3	0.06	0.17
4	0.08	0.22
5	0.25	0.57
6	0.23	0.45
7	0.12	0.13
8	0.11	0.28
9	0.09	0.36
Mean OD \pm S.E.	0.07 \pm 0.03	0.16 \pm 0.05
t paired	t = 4.37 p < 0.05 for 8 df	
Copositivity	15/15 = 100%	
Conegativity	5/9 = 55%	
Total	19/24 = 83%	

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set, with only a serum code number, was sent to the other laboratory for tests to be conducted blind. On completion the test results were compared with those obtained by the "reference" laboratory.

A set of data on a series of 24 matched samples, is presented in table 35. As the results show there was good agreement on positive samples between the two laboratories with copositivity rates of 100%. The agreement on negatives was less good indicating rather high 'test false positive reactions' in the assay performed in St. Lucia. This reduced the overall agreement in test performance to 83%. The paired t statistic was used to assess the degree of quantitative agreement in results between the two laboratories. As the results in table show there was a significant difference between OD levels for both positive and negative samples, but this was probably attributable to a few samples showing very wide OD variation. The same trend of high and low antibody levels was observed in the two sets of data.

Despite variability in quantitative readings and a proportion of false positive reactions in the negative samples screened in St. Lucia, possibly due to a higher non-specific background level in the conjugate used, the agreement between results was acceptable. Continued use of this type of exchanged material should provide a useful basis for establishing quality control in enzyme immunoassays.

0.09
df

0.05
f

CHAPTER IV

DISCUSSION AND SUMMARY

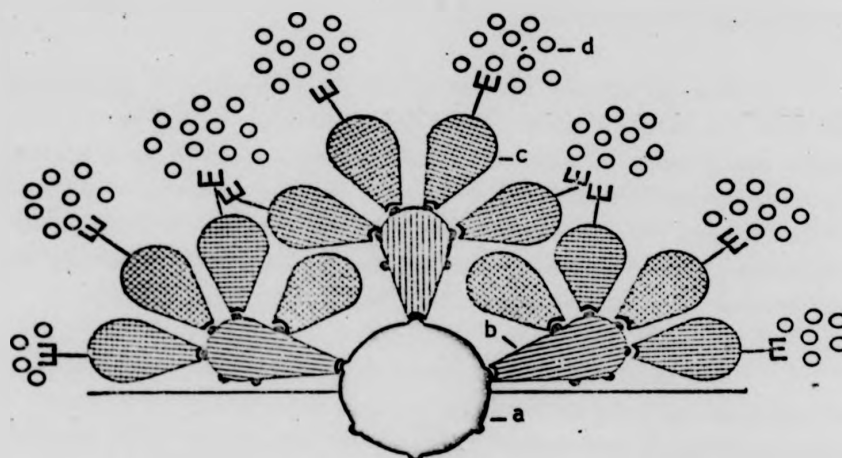
It could be argued that schistosomiasis has not presented a major diagnostic challenge to the immunologist, since infection can usually be diagnosed by parasitological means. The need to develop immunodiagnostic tests might therefore seem to be questionable, especially because of the lack of progress that has been made in this field over a substantial period of time. Although it is hard to dispute such views the studies described in this thesis hopefully provide some indications that immunodiagnosis can and should have a useful role in the epidemiology of schistosomiasis.

The definitive criterion for the diagnosis of S. mansoni infection is usually taken as the demonstration of ova by the microscopic examination of faeces. This has been regarded as a reliable approach for both the qualitative and quantitative determination of infection and has almost always provided the reference point for serological evaluation. Until recently most of the assumptions underlying the performance of parasitological tests have gone unchallenged.

The capacity of a diagnostic procedure to yield valid epidemiological data is based on several independent qualities; sample size, sensitivity and specificity. In a serological test sensitivity and specificity are dependent on both the intrinsic features of the assay system and the antigens used. The ELISA possesses a number of features which contribute to the development of a test with high sensitivity. In common with other labelled antibody techniques it possesses an "amplification" factor which directly enhances test sensitivity. This effect, which has been described by Nairn (1962), is due to the increase in combining sites provided by the middle antibody layer which acts as antigen for the enzyme antiglobulin conjugate. Furthermore, each enzyme molecule can hydrolyse several molecules of substrate thus increasing this effect. A diagram illustrating this principle is shown in Figure 10.

Another important feature of ELISA is its capacity to yield objective results based on a single dilution of test sera, a significant

Figure 10. Hypothetical explanation of enhanced sensitivity of indirect labelled antibody assays



- a - antigen
- b - antiserum
- c - enzyme antiglobulin conjugate
- d - enzyme substrate

(based on a diagram by Nairn, 1962)

improvement over most other serological methods which are based on the subjective assessment of an endpoint titration. The ability to obtain a direct measure of antibody activity in a serum sample however poses demands on test standardisation, and this is one of the most important yet least well developed aspects of enzyme immunoassays.

This work describes a new approach to end point standardisation, based on the degree of reactivity of carefully selected groups of serum samples read at predetermined absorbance values for the reference positive serum. This method overcomes the earlier problems of time based reaction rates which tended to produce high inter assay variation. The distribution of OD values at the selected end point of 0.75 also enabled diagnostic absorbance levels to be set. The correct way to express the amount of antibody activity measured in ELISA is, however, an equivocal point at present, and there is some controversy about the most meaningful form of expression.

The most commonly adopted methods reported in the literature are absorbance values at a single dilution or end point titres. The former method was selected in this study as it was particularly convenient for epidemiological surveys.

Although there have been some two hundred or more publications on the use of ELISA in serodiagnosis, surprisingly little attention has been focused on studies of test parameters. In this project an attempt was made to examine a number of important test components. The quality of the microtitre plates used in the test was demonstrated to be particularly important. Significant differences were encountered between irradiated and non-irradiated plates, possibly because of differences in their antigen uptake. Similar differences have been reported between polystyrene and polyvinyl plates (Voller, personal communication, 1979). In selecting a suitable grade of microtitre plate the chief consideration is to achieve uniform coating of antigen in each well, with an inter assay variation ideally of less than 10%, with minimal nonspecific binding of free antibody and conjugate. Problems were encountered with attempts to store precoated antigen plates. The main difficulty was the increase in nonspecific background reactivity which affected negative readings. This was possibly due to excess moisture seeping into the storage bags because of the difficulty in maintaining airtight conditions.

A more successful development for epidemiological surveys was the use of blood eluted from spots on absorbant paper. Tests showed no difference in the amount of antibody measured in the blood eluates compared with matched serum samples. However, there was some loss in antibody activity after long term storage of the papers. Following field collection samples should ideally be tested within a period of six months.

There has been little attempt to standardise serological assay procedures so that meaningful results can be obtained between different laboratories. The need to establish quality control procedures is of paramount importance in any test, but while many different techniques continue to be used along with poorly defined antigens the prospects for such standardisations are remote. The format adopted for the ELISA of plotting mean and standard deviations for replicate readings of a control positive serum provided a convenient method for assessing test reproducibility, particularly for tests performed on a routine basis. As the joint study carried out in St. Lucia later showed, quality control is essential in evaluating the performance of an assay system in two different laboratories.

The ELISA clearly has a number of features already given which make it a test worth exploiting for serodiagnosis. However, its capacity to yield sensitive and specific diagnostic information resides equally in the type of antigens used in the test. The antigens prepared in this study were essentially simple crude extracts of cercariae, worms and eggs following homogenisation. These types of preparations have generally been a recipe for failure in most of the earlier immunodiagnostic tests because they contain a high proportion of cross reactive antigens.

In ELISA microgram concentrations of protein are generally sufficient to provide an excess of antigen on the surface of each well in the microtitre plate, as illustrated in the calibration data. This is an important aspect with regard to test specificity. Although only crude antigen extracts were prepared for use in the test, by combining ultracentrifugation and subsequent dilutions up to two to three hundred times, a number of potentially cross reactive antigens could be removed. As table 2 outlining the biochemical composition of the antigens illustrates, some 50-70% of total protein was removed in the

first ultracentrifugation step. The importance of this is clearly shown in the epidemiological studies and will be discussed later.

The source of antigen used in the test is also important. As the studies clearly show soluble egg antigens are considerably more reactive than worm preparations, and can increase test sensitivity some 10-20%. This is not surprising since egg secretory products are a potent stimulus to the immune system and can induce strong delayed hypersensitivity responses. Further, unlike the adult worm, they are probably not coated in host proteins and therefore masked from the effects of the immune response.

The diagnostic importance of egg antigens has been shown in some of the more recent studies (Pelley *et al.*, 1977; Ruiz-Tiben *et al.*, 1979), and this development is of interest because of the almost exclusive use of worm antigen extracts in the early immunodiagnostic tests.

Before concluding the discussion of assay parameters it is important to draw attention to the differences that exist between the performance of different enzyme immunoassays for schistosomiasis. ELISA should be regarded as a generic name covering a range of different test procedures of often different quality. In a recent paper Hillyer *et al.* (1979a) reported an ELISA, using an egg antigen analogous to the one used in this study, to be an unsatisfactory sero-diagnostic procedure with low sensitivity (75%) and specificity. The actual test procedure used in their study differed in many respects from the one developed here, being characterised by very long incubation periods, 3 days at 4°C for antigen absorption and 18 hours for serum. There is no evidence that very long incubation of antigen is necessary, indeed some antigens may be lost with excessive incubation. Unless the concentration of antibody in the sample under test is very low a few hours (2-3) is generally sufficient to obtain immunological equilibrium between antigen and antibody. Long incubation periods are likely to increase the uptake of nonspecific immuno globulin hence reducing test specificity. Undoubtedly the most significant difference between the two assay systems is the choice of substrate. In their study Hillyer *et al.* (1979a) used 5-amino salicylic acid which is one of the least satisfactory peroxidase substrates having poor solubility and a very low dose response curve. Orthophenylenediamine (OPD) used in this study is not only highly soluble but is some 20-30 times more sensitive, giving a much greater ratio between positive and negative samples

(Voller et al., 1979).

These differences illustrate the importance of optimising an assay system and the ease with which a test procedure can be condemned as unsatisfactory simply because of inadequate evaluation of the different parameters.

In epidemiology lack of specificity in a test has probably been the main limitation to the use of immunodiagnostic methods. The term specificity has however often been rather loosely applied and its determination in immunodiagnostic tests has been based on such widely differing conditions as a) cross reactions from unrelated helminth infections, most commonly other helminths and avian and animal schistosomes, in people living in areas not endemic for human schistosomiasis and b) the measurement of antibody in individuals living in endemic schistosome areas in whom ova are not found. This comprises both the measurement of antibody, in individuals with a present active infection misdiagnosed parasitologically, and the measurement of residual antibody from previous infections. These are quite distinct conditions and yet in many studies only one has been used as the definitive criterion for determining test specificity. In this thesis a fairly dogmatic approach has been adopted. Condition (a) is regarded as the most important and the true criterion of test specificity since it represents the only condition in which schistosome infection can be reliably excluded.

The studies described in Chapter 4 were designed to assess the degree of cross reactivity in the ELISA amongst individuals without schistosomiasis, and to relate the specificity to the levels previously determined by some other conventional immunological methods. As the results showed ELISA is considerably more specific than skin tests, CFT and IFAT in discriminating between schistosome infections and other helminths.

Substantial reductions in cross reactivity were also observed in ELISA in a group of patients with cercarial dermatitis which had produced very high levels of reactivity, over 60%, in the other tests. This represents an important development, as in the past the use of immunological methods has been limited in those areas where bird and animal schistosomes are prevalent. The capacity of the test to distinguish between species of schistosomes was weak, with cross reactions occurring in a high percentage of patients infected with

S. haematobium.

Overall results of these studies show the ELISA, even using crude antigen preparations, to be a sufficiently specific test to exclude the majority of helminth infections which have produced extensive cross reactions in most other tests used in epidemiological surveys in the past. Although condition (b) has been widely used as a determinant of specificity it provides an unreliable measure because it is dependent on the quality of parasitological diagnostic methods for excluding the presence of schistosome infection.

Parasitological diagnostic methods for schistosomiasis are theoretically specific in that they should not yield false positives but their sensitivity is variable depending on factors such as age, prevalence and associated intensity and the effective size of the stool or urine sample used in the test. This concept is important in studies to evaluate serological methods and has been well expressed by Ruiz-Tiben et al. (1979):

"if the reference classification is used to measure the capacity of a screening test to differentiate infected and non-infected individuals, the observed results will simply reflect the relative agreement between the screening test and the reference classification. For these reasons, the relative sensitivity of stool or urine examination tests in detecting infection in subjects with low levels of egg output is of crucial importance in studies where such tests are used to establish the parasitologic status of individuals for comparison with results of immunodiagnostic techniques."

The detection of antibody in individuals negative parasitologically is clearly an important aspect of serological test specificity, as it impinges on the whole question of using parasitological diagnostic methods as reference tests. The probability of detecting infection parasitologically is strongly dependent on the intensity of infection in a community. This is important since the schistosome worm does not multiply within the definitive host and the intensity profile within a community is generally skewed with only a small proportion of people excreting large numbers of eggs. The sensitivity of a serological test on the other hand, is less influenced by infection intensity. Since antibody production is a homeostatically controlled process, individuals receiving sufficient antigenic challenge should produce detectable levels of antibody. The results

of tests to determine ELISA sensitivity illustrate this point clearly. In both the Sudanese and St. Lucia studies antibodies were detected with the egg antigen in almost all individuals diagnosed parasitologically. This included individuals in groups with very low egg counts, 10-25 epg, at the threshold of detection of the parasitological techniques.

The relationship between the sensitivity and specificity of parasitological and serological tests is also governed by the true prevalence of infection in an endemic area. Buck & Gart (1966), have formulated an interesting model relating the "cpositivity" and "conegativity" of a reference and screening test, to changes in the true prevalence of infection. They have postulated an inverse relationship, with copositivity increasing as the prevalence of infection increases and conegativity decreasing, and vice versa. The situations in the Gezira region of the Sudan and St. Lucia illustrate these contrasting conditions. In St. Lucia the control programme has resulted in marked decreases in prevalence as a consequence of which one would expect the disparity in copositivity between parasitological and serological tests to increase.

The effect of decreases in prevalence and intensity on the capacity of parasitological methods to detect infected individuals has been studied by Ruiz-Tiben *et al.* (1979) in infected communities in Puerto Rico. The island is in many ways analogous to St. Lucia in terms of endemic S. mansoni infection characterised by low prevalence and intensity of infection (mean of 3.6 epg). In their study to assess the diagnostic validity of a number of serological tests, a significant relationship was observed between the number of stools examined and the sensitivity and specificity of the serological tests. The specificity of the COPT increased from 66% when compared with a single stool examination to 96% when multiple stool examinations were carried out. In their study the authors used a reputedly sensitive parasitological technique, the Modified Ritchie formol ether concentration technique (MRCT) and postulated that if a less sensitive method such as the Kato, which is commonly used in epidemiological studies, had been used over 50% of the infected population would have been misdiagnosed.

The implications of this study have a direct bearing on the studies carried out in St. Lucia. Here routine diagnosis of infection

is based upon the examination of 1 gm of a single stool specimen processed by the Bell technique. As the results of the comparative study on the two serological and parasitological techniques have shown the Bell is a far from satisfactory diagnostic method when used routinely, with almost 1 in 7 individuals being misdiagnosed. Although false negatives were anticipated the high proportion of false positives by stool examination was surprising and this is possibly one of the first studies where this phenomenon has been reported. Mention has been made of the possible causes, the most likely being contamination of the apparatus. The point that is worth making is that while parasitological diagnosis is inherently very specific, under routine practical conditions false positives can occur and will be most evident where prevalence rates are decreasing or very low. The poor performance of the Bell technique in this study might be an exception in routine diagnosis, but it does challenge the almost universal acceptance of parasitological diagnosis as a reference method. An important difference of the comparative study in this project was the decision to use a combination of parasitological and serological results as a diagnostic reference. Although an unorthodox approach it was an attempt to remove the 'inherent bias' in favour of parasitological tests, to treat each test equally and still provide a logically acceptable reference result. The decision to incorporate serology was felt to be justified since both RIA and ELISA had emerged with acceptable levels of sensitivity and specificity in earlier studies. Another important difference was that, with the exception of the Kato test, all other tests were performed by locally trained staff and therefore represented conditions experienced in a laboratory in an endemic area.

As the results of the study showed, there were marked differences in the sensitivity and specificity of the tests with the two serological tests emerging as the most sensitive but with apparent high false positive rates of over 20%. This illustrates one of the weaknesses of the chosen diagnostic reference. Although both serology and parasitology were combined to form a reference test the examination of only a single stool specimen undoubtedly affected the level of sensitivity of the parasitological tests and hence the reference, thus influencing the "specificity" of the serological tests. In order to determine true infection status multiple stool examinations should have been carried out as in an earlier study. This showed a high proportion of individuals diagnosed as infected by both ELISA and RIA but negative parasitologically, to be infected when several stool samples were again

examined.

Clearly as these studies have shown, the presence of antibody in individuals parasitologically negative is not necessarily a false positive reaction and therefore related to specificity but may simply reflect the differences in sensitivity between the parasitological and serological tests. This point is raised by Hillyer et al. (1979b) "In an endemic area such as Puerto-Rico some persons will still be negative after multiple stool examinations but may be serologically positive if the test is sufficiently sensitive. Thus in an endemic area, specificity will approach 100% but never reach it." This raises the important question of how to reliably evaluate the performance of a serological test particularly in low prevalence areas like Puerto Rico and St. Lucia.

Another parasitological diagnostic problem that is caused by a decrease in prevalence is the tedium associated with technicians having to examine large numbers of negative stool specimens, a problem which has been stressed by Goddard (1977). "In routine survey work for schistosomiasis there needs to be a substantial laboratory to process samples for deriving even the simplest measure of infection in a population, that of prevalence. Indeed as prevalence and other indices drop diagnostic imperfection may play an important role."

A serological test such as ELISA, which is streamlined to process large numbers of samples, could overcome this problem. At present one technician can process about 60 stool samples per week by the Bell technique, and this could be increased to between 500-1,000 by ELISA. Quality control could also be implemented much more easily in serodiagnostic testing. The problem of misdiagnosis by parasitological methods also has important implications in incidence monitoring. Changes in the incidence rate provide the most sensitive indicator of changes in transmission and are the most commonly adopted epidemiological parameters for assessing the efficacy of control schemes. It demands a diagnostic procedure of high sensitivity so that uninfected individuals can be reliably selected and subsequent conversion measured. The greater sensitivity of serodiagnostic procedures such as ELISA and RIA compared to conventional parasitological methods makes these techniques ideally suited for incidence determinations.

The second point mentioned in the context of test specificity with respect to condition (b), the measurement of residual antibody in an individual from a previous infection, is not a measure of the degree of specificity of a serological test but rather of its capacity to differentiate period from point prevalence. The essential difference in these two measurements is clearly illustrated in the age specific cross sectional prevalence analysis of the Bor and Gezira areas in the Sudan and in the study in St. Lucia.

The serological and parasitological prevalence rates in children and adolescents are more or less parallel, but tend to diverge in the older age groups. This difference clearly has important practical considerations. In children and adolescents, generally the most important groups with regard to control strategy, serology can provide valid diagnostic information of infection status and may, as already discussed, have a special role in incidence determination.

In adults the value of serology is perhaps less clear. In the Sudan the prevalence of antibodies in adults remains high and probably reflects repeated exposure to infection. In St. Lucia there is a drop in antibody prevalence in older individuals, which may relate to the loss of infections and/or absence of re-exposure. One interesting feature to emerge from both studies was the absence of an increase in antibody levels in the older individuals despite continued exposure. This finding which has been reported by other groups is postulated by Nash (1978) to represent some form of humoral modulation. It represents an interesting contrast to the situation observed in endemic malaria. Here repeated exposure results in an increase in antibody levels as functional protective immunity develops (Draper et al. 1972).

The period prevalence rate has not been widely used as an epidemiological measure in schistosomiasis, unlike malaria where it has proved particularly valuable in retrospective studies. However with good test specificity so that only anti-schistosomal antibody is measured, period prevalence could become useful, particularly as an indicator of the frequency of exposure and for obtaining baseline data prior to the start of a control programme.

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A narrow perspective, that of test specificity, has been

used to assess the value of serology for epidemiological monitoring of schistosomiasis. The equivocal nature of its determination has undoubtedly influenced the use of serological tests, and this makes it an important concept in qualitative diagnosis. As the studies have illustrated, there are strong arguments in favour of using sera from carefully chosen populations in non-endemic areas as the sole condition for determining serological test specificity. In schistosome endemic areas serological specificity cannot be reliably measured because of its dependence on the sensitivity of parasitological diagnostic methods. This becomes critical with tests such as ELISA and RIA which have higher sensitivity thresholds than most parasitological tests used for routine diagnosis. Clearly some form of sensitivity index, such as that developed by Ruiz-Tiben et al. (1979) which relates serological positivity based on a single measurement with parasitological positivity rates based on multiple stool examinations is needed for proper evaluation.

What has just been discussed has centred on the basic "qualitative" epidemiological applications of serology, dependent on the levels of sensitivity and specificity in the test, and the practical considerations for routine use. Possibly of equal importance is the need to obtain quantitative information on communities infected with S. mansoni, particularly for morbidity studies and for assessing the responses to chemotherapy. A number of studies to assess the value of ELISA for determining quantitative epidemiological parameters were undertaken, but although the results were interesting, little in the way of practical applications has emerged as yet. There was no striking relationship between antibodies measured to soluble egg antigen (SEA) and egg count, at present the most valid epidemiological measure of infection intensity. In the Sudanese studies very low correlation coefficients were observed between total anti-SEA antibody levels and infection intensity in a wide range of age groups. A better correlation was observed in the St. Lucia surveys when monospecific IgG conjugates were used, but the association was too weak to be put to practical use.

The capacity of ELISA to measure the different classes of antibody is a feature of the test which could be exploited, although at present the function of particular antibody species in S. mansoni infections is far from clear. Measurements of specific IgM antibody

in the St. Lucia studies were very inconclusive. A high proportion of infected children had detectable IgM and this decreased in the adult population, suggesting that specific IgM may be more associated with early infection, a characteristic feature of many other infectious diseases.

Studies from other groups tend to confirm these findings. Deelder et al. (1975b) demonstrated the presence of specific IgG, M, and A antibodies in almost all patients infected with S. mansoni, although as the authors stressed their role was unclear. In a more recent study Kanamura et al. (1978) again detected the presence of all classes of specific antibody by IFAT and found in particular that IgA was associated with the "acute" phase of infection.

While crude poorly defined antigens are used in serological assays, the function of humoral responses in schistosomiasis is unlikely to be resolved. The study of antibody levels following chemotherapy illustrates this clearly. While there is good evidence for the loss of antibody activity to SEA following effective treatment, until the appropriate antigen which stimulates the production of antibody with a short half life is isolated, the possibility of monitoring the efficacy of treatment on a routine basis is at present limited. Similarly there was no observed difference in antibody levels between patients with hepatosplenic S. mansoni infection and carefully matched controls. These results do not however rule out the involvement of antibodies in the modulation of the granuloma response.

This thesis has described the work carried out to investigate the serodiagnostic properties of ELISA, in a number of epidemiological applications. It has been carried out in a field which has received much criticism over a fairly long period of time, and which many have felt has no useful place in the epidemiology of S. mansoni infections. The studies presented here have hopefully gone some way to refute these views, while at the same time acknowledging that problems still exist. Progress in this field has certainly been very slow, but the main problems to the use of serodiagnostic methods are now being resolved and the future can be regarded with optimism. Although initial emphasis is likely to be placed on the use of antibody measurements for epidemiological surveys, it would be wrong to ignore the potential of antigen detection. Although still in its infancy it

may eventually be possible to detect antigen in significant proportions, using sensitive techniques like ELISA or RIA, and to expand further the use of immunological indices in community studies.

In summarising the main points, ELISA has been shown to be a useful assay procedure for S. mansoni infection. It has a number of features which make it eminently suitable for exploitation in epidemiological studies. Comparative studies with parasitological techniques have shown that in many cases serology can provide equally relevant information but more accurately and economically. The diagnostic problems generated by decreasing prevalence offer a unique opportunity to use serological procedures such as ELISA, and throw open to question the viability of parasitological methods both as reference tests and as routine diagnostic tests. The main weakness to emerge from these studies covers the use of ELISA for quantitative epidemiological measurements, but as other studies using purified antigens have shown these problems may be resolved in time.

CHAPTER V

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Appendices

1 Statistical tests

The following standard statistical procedures were used:

Frequency distributions, calculations of means and standard errors based on conversion of data into working units.

Variance ratio (F) test

Unpaired and Paired t test

Regression analysis and measurement of correlation coefficient

Cochrane's Q analysis

Most statistical tests were carried out on a Hewlett Packard Model 10 calculator using standard programmes. Data from the Sudanese survey were processed by the Department of Medical Statistics, L.S.H. & T.M.

2 Buffers for ELISA

Coating buffer 0.05M carbonate pH 9.6

1.59 g Na_2CO_3

2.93 g NaHCO_3

(0.2 g NaN_3)

Dissolved in 1 litre distilled water

Incubation buffer

900 ml PBS

0.45 ml Tween 20

(0.18 g NaN_3)

Elution buffer

900 ml PBS

0.45 ml Tween 20

1% BSA

Washing buffer

45 g NaCl

2.5 ml Tween 20

diluted to 5 l. with distilled water

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Enzyme labelled anti-globulin conjugates

In addition to laboratory prepared conjugates a number of commercially prepared conjugates were found to be satisfactory. They were as follows:

Miles Laboratories - peroxidase labelled anti-human IgG, IgM, IgA conjugates.

Dynatech Laboratories - Phosphatase labelled anti-human IgG conjugate

Phosphatase substrate

5 mg tablet pNPP (Sigma)

dissolved in 5 ml diethanolamine

Diethanolamine buffer pH 9.8

97 ml diethanolamine

0.5 mM MgCl_2 - 101 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

0.2 g NaN_3

800 ml distilled water

5-Amino salicylic acid stock

8 mg 5-ASA dissolved in 10 ml warm distilled water

Working solution

To an aliquot of stock soln. 1M NaOH added to final pH 6.0

To 1.0 ml of resultant solution add 1.0 ml 0.05% H_2O_2 .

Ortho-Phenylene diamine stock

100 mg OPD dissolved in 10 ml methanol.

Stored in the dark at 4°C for 1 week

Working solution

1.0 ml stock added to 99 ml distilled water and 0.05 ml 6% solution of H_2O_2

Substrate inhibitors

Phosphatase 3M NaOH

Peroxidase 8N H_2SO_4